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(54) **METHODS FOR REPROGRAMMING CELLS AND USES THEREOF**

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(57) **ABSTRACT**

Described herein are methods for cell dedifferentiation, transformation and eukaryotic cell reprogramming. Also descried are cells, cell lines, and tissues that can be transplanted in a patient after steps of in vitro dedifferentiation and in vitro reprogramming. In particular embodiments the cells are Stem-Like Cells (SLCs), including Neural Stem-Like Cells (NSLCs). Also described are methods for generating these cells from human somatic cells and other types of cells. Also provided are compositions and methods of using of the cells so generated in human therapy and in other areas.

5 Claims, No Drawings

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METHODS FOR REPROGRAMMING CELLS AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to the field of eukaryotic cell reprogramming, and particularly to cell dedifferentiation. The invention is also concerned with methods of generating stable Neural Stem-Like Cells (NSLCs) from human somatic cells (and other cells) and the use of the cells so generated in human therapy.

BACKGROUND OF THE INVENTION

Cell Reprogramming

There is a desire in the medical, scientific, and diagnostic fields to reprogram an easily obtainable cell into a cell that is generally harder to obtain, or to reprogram a cell to have new or different functionalities, without fusing or exchanging material with an oocyte or another stem cell.

According to a first mechanism, a stem cell can naturally divide or differentiate into another stem cell, progenitor, precursor, or somatic cell. According to a second mechanism, somatic cell can sometimes transiently change its phenotype or express certain markers when placed in certain conditions, and then revert back when placed back into the original conditions. According to a second mechanism, the phenotype of many cells can be changed through forced expression of certain genes (for example, stably transfecting the c-myc gene into fibroblasts turns them into immortal cells having neuroprogenitor characteristics), however once this forced gene expression is removed, the cells slowly revert back to their original state. Therefore, none of the three above mechanisms should be considered true reprogramming: the first is considered natural differentiation which is part of a cell program that is already in place (going from a more undifferentiated to a more differentiated state), the second is a transient phenotypical change, and the third is a constantly forced cell type. A true stem cell: (i) self-renews almost 'indefinitely' (for significantly longer than a somatic cell), (ii) is not a cancerous cell, (iii) is not artificially maintained by forced gene expression or similar means (must also be able to be maintained in standard stem cell media), (iv) can differentiate to progenitor, precursor, somatic or other more differentiated cell type (of the same lineage), and (v) has all the characteristics of a stem cell and not just certain markers or gene expression or morphological appearance.

Despite the numerous scientific and patent publications claiming successful reprogramming or dedifferentiation, generally into a stem cell, almost all of these publications do not disclose true reprogramming because they fall under one of the mechanisms mentioned above. For instance, Bhasin (WO2010/088735), Cifarelli et al. (US2010/0003223), Kremer et al. (US2004/0009595), and Whinier et al. (US2010/0047908) all refer to reprogramming, dedifferentiation, and/or obtained stem cells (or progenitors) as phenotypical cell changes based only on a change in cell surface markers after culture in different media with supplements, with no evidence of true reprogramming or an actual stem cell (non-cancerous self-renewal with stem cells markers and no differentiation markers). The same is true for Benneti (WO2009/079007) who used increased expression of Oct4 and Sox2. Others, such as Akamatsu et al. (WO2010/052904) and You et al. (WO2007/097494, US2009/0246870), refer to having made stem cells, but these came about through constant artificial gene induction delivered by retrovirus (similar to cMyc) with no evidence of true stem cells that are not immortal/tumorigenic, and stable instead of transient. Others, such as Chen et al. (US2005/0176707) and You et al. (US2009/0227023), have made "multipotent cells", but not stem cells. In addition these alleged multipotent cells were not stable (in the case of You et al. the cells could not even proliferate) and both used constant media supplements and conditions to force the phenotypical change. Yet others, such as Oliveri et al. (WO2009/018832) and Zahner et al. (US2002/0136709), have claimed the making of pluripotent, totipotent, multipotent, and/or unipotent cells automatically through genome-wide DNA demethylation and histone acetylation, but with no evidence of a stable, non-cancerous, true cell line.

True reprogramming appears to have been achieved with induced pluripotent stem cells (iPS cells) created independently by Yamanaka's group (Takahashi et al., 2007) and Thomson's group (Yu et al., 2007), and potentially by others before them, and although many of these cells were later found to be cancerous, some of them were not. These cells can be induced by true reprogramming since it was later shown that they can also be induced by non-gene integrating transient transfection (Soldner et al., 2009; Woltjen et al., 2009; Yu et al., 2009) as well as by RNA (Warren et al., 2010) or protein (Kim et al., 2009; Zhou et al., 2009) alone or by small molecules (Lyssiotis et al., 2009), and by similar methods. However, these cells are essentially identical to embryonic stem cells and have the same problems of uncontrolled growth, teratoma formation, and potential tumor formation.

A more desirable option is to have multipotent stem cells or pluripotent-like cells whose lineage and differentiation potential is more restricted so that they do not readily form teratomas and uncontrolled growth. There is thus a need for methods of creating multipotent stem cells, multipotent stem-like cells, and stem-like cells and method of reprogramming or transforming easily obtainable cells to highly desirable multipotent stem cells, multipotent stem-like cells, and stem-like cells.

Neural Stem-Like Cells (NSLC)

Repairing the central nervous system (CNS) is one of the frontiers that medical science has yet to conquer. Conditions such as Alzheimer's disease, Parkinson's disease, and stroke can have devastating consequences for those who are afflicted. A central hope for these conditions is to develop cell populations that can reconstitute the neural network, and bring the functions of the nervous system back in line. For this reason, there is a great deal of evolving interest in neural stem and progenitor cells. Up until the present time, it was generally thought that multipotent neural progenitor cells commit early in the differentiation pathway to either neural restricted cells or glia restricted cells.

Neural stem cells have promise for tissue regeneration from disease or injury; however, such therapies will require precise control over cell function to create the necessary cell types. There is not yet a complete understanding of the mechanisms that regulate cell proliferation and differentiation, and it is thus difficult to fully explore the plasticity of neural stem cell population derived from any given region of the brain or developing fetus.

The CNS, traditionally believed to have limited regenerative capabilities, retains a limited number of neural stem cells in adulthood, particularly in the dentate gyrus of the hippocampus and the subventricular zone that replenishes olfactory bulb neurons (Singec I et al., 2007; Zielton R, 2008). The availability of precursor cells is a key prerequisite for a transplant-based repair of defects in the mature nervous system. Thus, donor cells for neural transplants are largely derived from the fetal brain. This creates enormous ethical problems, in addition to immuno-rejection, and it is questionable whether such an approach can be used for the treatment of a large number of patients since neural stem cells can lose some of their potency with each cell division.

Neural stem cells provide promising therapeutic potential for cell-replacement therapies in neurodegenerative disease (Mimeault et al., 2007). To date, numerous therapeutic transplantations have been performed exploiting various types of human fetal tissue as the source of donor material. However, ethical and practical considerations and their inaccessibility limit the availability as a cell source for transplantation therapies (Ninomiy M et al., 2006).

To overcome barriers and limitations to the derivation of patient specific cells, one approach has been to use skin cells and inducing the trans-differentiation to neural stem cells and/or to neurons (Levesque et al., 2000). Transdifferentiation has been receiving increasing attention during the past years, and trans-differentiation of mammalian cells has been achieved in co-culture or by manipulation of cell culture conditions. Alteration of cell fate can be induced artificially in vitro by treatment of cell cultures with microfilament inhibitors (Shea et al., 1990), hormones (Yeomans et al., 1976), and Calcium-ionophores (Shea, 1990; Sato et al., 1991). Mammalian epithelial cells can be induced to acquire muscle-like shape and function (Paterson and Rudland, 1985), pancreatic exocrine duct cells can acquire an insulin-secreting endocrine phenotype (Bouwens, 1998a, b), and bone marrow stem cells can be differentiated into liver cells (Theise et al., 2000) and into neuronal cells (Woodbury et al., 2000). Other such as Page et al. (US 2003/0059939) have transdifferentiated somatic cells to neuronal cells by culturing somatic cells in the presence of cytoskeletal, acetylation, and methylation inhibitors, but after withdrawal of the priming agent, neuron morphology and established synapses last for not much than a few weeks in vitro, and complete conversion to a fully functional and stable type of neuron has never been demonstrated. These are thus transient cell phenotypes. Complete conversion to a fully functional and stable type of neuroprogenitor or neural stem cell has also never been demonstrated. Acquisition of a stable phenotype following transdifferentiation has been one of the major challenges facing the field.

Thus, there is a need in the biomedical field for stable, potent, and preferably autologouos neural stem cells, neural progenitor cells, as well as neurons and glial cells for use in the treatment of various neurological disorders and diseases. The same is true for many other types of cells. Recently, evidence have been obtained that genes of the basic Helix-Loop-Helix (bHLH) class are important regulators of several steps in neural lineage development, and over-expression of several neurogenic bHLH factors results in conversion of non-determined ectoderm into neuronal tissue. Proneural bHLH proteins control the differentiation into progenitor cells and their progression through the neurogenic program throughout the nervous system (Bertrand et al., 2002). MASH1, NeuroD, NeuroD2, MATH1-3, and Neurogenin 1-3 are bHLH transcription factors expressed during mammalian neuronal determination and differentiation (Johnson et al., 1990; Takebyashi et al., 1997; McCormick et al., 1996; Akazawa et al., 1995). Targeted disruptions of MASH1, Ngn1, Ngn2 or NeuroD in mice lead to the loss of specific subsets of neurons (Guillemot et al., 1993; Fode et al., 1998; Miyata et al., 1999).

U.S. Pat. No. 6,087,168 (Levesque et al.,) describes a method for converting or transdifferentiating epidermal basal cells into viable neurons. In one example, this method comprises the transfection of the epidermal cells with one or more expression vector(s) containing at least one cDNA encoding for a neurogenic transcription factor responsible for neural differentiation. Suitable cDNAs include: basic-helix-loop-helix activators, such as NeuroD1, NeuroD2, ASH1, and zinc-finger type activators, such as Zic3, and MyT1. The transfection step was followed by adding at least one antisense oligonucleotide known to suppress neuronal differentiation to the growth medium, such as the human MSX1 gene and/or the human HES1 gene (or non-human, homologous counterparts). Finally, the transfected cells were grown in the presence of a retinoid and a least one neurotrophin or cytokine, such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), or neurotrophin 4 (NT-4). This technology yields 26% of neuronal cells; however, neither functionality nor stability of these cells was established. In addition, neural stem cells or neuroprogenitor cells are not produced according to this method.

A later process (Levesque et al., 2005; U.S. Pat. No. 6,949,380) mentions the conversion of the epidermal basal cell into a neural progenitor, neuronal, or glial cell by exposing the epidermal basal cell to an antagonist of bone morphogenetic protein (BMP) and growing the cell in the presence of at least one antisense oligonucleotide comprising a segment of a MSX 1 gene and/or HES1 gene. However, there is no evidence or examples that any neural progenitors or glial cells were produced according to this method, let alone any details or evidence that morphological, physiological or immunological features of neuronal cells was achieved. In addition, since there is also no information on functionality, stability, expansion, and yield about the cells which may or may not have been produced, it is possible that these cells actually are skin-derived precursor cells (Fernandes et al., 2004) that have been differentiated into neuronal cells.

In view of the above, there is thus a need for stable, potent, and preferably autologouos neural stem cells, neural progenitor cells, neurons and glial cells, as well as other types of cells, stem cells and progenitor cells. There is also a need for methods that could result in true cell dedifferentiation and cell reprogramming.

The present invention addresses these needs and provides various types of stem-like and progenitor-like cells and cells derived or differentiated from these stem-like or progenitor-like cells, as well as methods that can result in true cell dedifferentiation and cell reprogramming.

Additional features of the invention will be apparent from a review of the disclosure and description of the invention herein.

SUMMARY OF THE INVENTION

The present invention relates to stem-like and progenitor-like cells and cells derived or differentiated from these stem-like or progenitor-like cells. The invention further relates to methods for cell dedifferentiation and cell reprogramming. The invention further features compositions and methods that are useful for reprogramming cells and related therapeutic compositions and methods.

One particular aspect relates to the development of a technology to reprogram a somatic cell or non-neuronal cell to a cell having one or more morphological physiological, and/or immunological features of a neural stem cell and which possess the capacity to differentiate along neuronal and glial lineages. According to some embodiments, the invention is more particularly concerned with methods of generating stable Neural Stem-Like Cells (NSLCs) from human somatic cells, human progenitor cells and/or of human stem cells, as well as cells, cell lines and tissues obtained by using such methods.

The invention further relates to compositions and methods to induce de-differentiation of human somatic cells into Neural Stem-Like Cells that express neural stem cell specific markers. According to the present invention it is possible to effect the conversion of cells to various types of differentiated neuronal cells that can be created from a single cell type taken from an individual donor and then reprogrammed and transplanted into the same individual. Upon induction cells according to the invention express neural stem-cell specific markers and become Neural Stem-Like cells.

According to one particular aspect, the invention relates to a method of transforming a cell of a first type to a desired cell of a different type. The comprises i) obtaining a cell of a first type; ii) transiently increasing in the cell of a first type intracellular levels of at least one reprogramming agent, whereby the transient increase induces direct or indirect endogenous expression of at least one gene regulator; iii) placing the cell in conditions for supporting the growth and/or the transformation of the desired cell and maintaining intracellular levels of the at least one reprogramming agent for a sufficient period of time to allow stable expression of the at least one gene regulator in absence of the reprogramming agent; and iv) maintaining the cell in culture conditions supporting the growth and/or the transformation of the desired cell. Such conditions are maintained for a sufficient period of time to allow a stable expression of a plurality of secondary genes. According to the invention the expression of one or more of the secondary genes is characteristic of phenotypical and functional properties of the desired cell while being not characteristic of phenotypical and functional properties of an embryonic stem cell. Therefore, at the end of the period of time, the desired cell of a different type is obtained.

According to another particular aspect, the invention relates to a method of transforming a cell of a first type to a cell of a second different type. The method comprises contacting the cell of a first type with one or more agents capable of increasing within said cell levels of at least one reprogramming agent and directly or indirectly remodeling the chromatin and/or DNA of the cell. The at least one reprogramming agent is selected for inducing directly or indirectly the expression of morphological and functional characteristics of a desired cell of a different type or different cell lineage.

According to another aspect, the invention relates to a method of transforming a cell of a first type to a cell of a second different type. The method comprises contacting the chromatin and/or DNA of a cell of a first type with an agent capable of remodeling chromatin and/or DNA of said cell; and increasing intracellular levels of at least one reprogramming agent. The at least one reprogramming agent is selected for inducing directly or indirectly the expression of morphological and functional characteristics of a desired cell of a different type or cell lineage.

A further aspect of the invention relates to a method of transforming a cell of a first type to a cell of a desired cell of a different type, comprising increasing intracellular levels of at least one reprogramming agent, wherein the at least one reprogramming agent is selected for inducing directly or indirectly the expression of morphological and functional characteristics of a desired second cell type; and maintaining the cell of a first type in culture conditions for supporting the transformation of the desired cell for a sufficient period of time to allow stable expression of a plurality of secondary genes whose expression is characteristic of phenotypical and functional properties of the desired cell, wherein at least one of the secondary genes is not characteristic of phenotypical and functional properties of an embryonic stem cell. At the end of the period of time the desired cell of a different type is obtained and the obtained cell is further characterized by a stable repression of a plurality of genes expressed in the first cell type.

A further aspect of the invention concerns a process wherein a cell of a first type is reprogrammed to a desired cell of a different type, the process comprising:

- a transient increase of intracellular levels of at least one reprogramming agent, wherein the at least one reprogramming agent induces a direct or indirect endogenous expression of at least one gene regulator, and wherein the endogenous expression of the said at least one gene regulator is necessary for the existence of the desired cell of a different type;
- a stable expression of said at least one gene regulator;
- stable expression of a plurality of secondary genes, wherein the stable expression of the secondary genes is the result of the stable expression of the at least one gene regulator, and wherein: (i) stable expression of the plurality of secondary genes is characteristic of phenotypical and/or functional properties of the desired cell, (ii) stable expression of at least one of said secondary genes is not characteristic of phenotypical and functional properties of an embryonic stem cell, and wherein (i) and (ii) are indicative of successful reprogramming of the cell of the first type to the desired cell of the different type.

In particular embodiments, the at least one reprogramming agent in the process is a Msi1 polypeptide, or a Ngn2 polypeptide together with a MDB2 polypeptide. In particular embodiments, the at least one gene regulator is Sox2 Msi1, or both. In additional embodiments the at least one gene regulator may is one or more of the genes listed in Table A for Neural Stem-Like Cells.

According to another aspect, the invention relates to a method of obtaining a Stem-Like Cell (SLC), comprising:

- i) providing a cell of a first type;
- ii) transiently increasing in the cell intracellular levels of at least one reprogramming agent, whereby the transient increase induces direct or indirect endogenous expression of at least one gene regulator;
- iii) placing the cell in conditions for supporting the transformation into the stem-like cell and maintaining intracellular levels of the at least one reprogramming agent for a sufficient period of time to allow stable expression of the at least one gene regulator in absence of the reprogramming agent;
- iv) maintaining the cell in culture conditions for supporting the transformation into the stem-like cell for a sufficient period of time to allow stable expression of a plurality of secondary genes whose expression is characteristic of phenotypical and/or functional properties of the stem-like cell, wherein at least one of the secondary genes is not characteristic of phenotypical and functional properties of an embryonic stem cell. At the end of said period of time a stem-like cell is obtained.

According to another aspect, the invention relates to a method of obtaining a Stem-Like Cell. The method comprises increasing intracellular levels of at least one polypeptide specific to the desired stem cell type that is able to drive directly or indirectly transformation of the cell of the first type into the Stem-Like Cell. For increasing the yield or type of Stem-Like Cell, the method may further comprises contacting chromatin and/or DNA of a cell of a first type with a histone acetylator, an inhibitor of histone deacetylation, a DNA demethylator, and/or an inhibitor of DNA methylation; and/or increasing intracellular levels of at least one other polypeptide specific to the desired stem cell type that is able to drive directly or indirectly transformation of the cell of the first type into a Stem-Like Cell.

According to another aspect, the invention relates to a method of obtaining a Neural Stem-Like Cell (NSLC). The method comprises increasing intracellular levels of at least one neural stem cell specific polypeptide that is able to drive directly or indirectly transformation of the cell of the first type into a NSLC. For increasing the yield or type of NSLC, the method further comprises contacting chromatin and/or DNA of a cell of a first type with a histone acetylator, an inhibitor of histone deacetylation, a DNA demethylator, and/or an inhibitor of DNA methylation; and/or increasing intracellular levels of at least one other neural stem cell specific polypeptide that is able to drive directly or indirectly transformation of the cell of the first type into a NSLC.

Another aspect of the invention concerns a method of obtaining a Neural Stem-Like Cell (NSLC). In one embodiment the method comprises transfecting a skin cell with a polynucleotide encoding Musashi1, Musashi1 and Neurogenin 2, Musashi1 and Methyl-CpG Binding Domain Protein 2 (MBD2), or Neurogenin 2 and Methyl-CpG Binding Domain Protein 2, thereby reprogramming the skin cell into a NSLC. In another embodiment the method comprises exposing a skin cell to: (i) an inhibitor of histone deacetylation, (ii) an inhibitor of DNA methylation, (iii) a historic acetylator, and/or (iv) a DNA demethylator such as a MBD2 polypeptide and/or transfecting with a polynucleotide encoding a MBD2 polypeptide; and further transfecting the cell (either simultaneously, before, or afterwards) with a polynucleotide encoding MUSASHI1 and/or with a polynucleotide encoding NGN2, thereby reprogramming the skin cell into a NSLC. Some other cells, such as keratinocytes and CD34 cells, can also be used and reprogrammed.

In one particular embodiment, the method of obtaining a Neural Stem-Like Cell (NSLC), comprises:

- providing a cell of a first type;
- introducing into the cell one or more polynucleotide capable of transient expression of one or more the following polypeptides: Musashi1 (Msi1); a Musashi1 (Msi1) and a Neurogenin 2 (Ngn2); a Musashi1 (Msi1) and methyl-CpG binding domain protein 2 (MBD2); and Neurogenin 2 (Ngn2) and methyl-CpG binding domain protein 2 (MBD2); and
- placing the cell in culture conditions supporting the transformation into a NSLC for a sufficient period of time to allow a stable expression of a plurality of genes whose expression is characteristic of phenotypical and functional properties of a NSLC.

At the end of the period of time a NSLC is obtained and the obtained NSLC is further characterized by a stable repression of a plurality of genes expressed in the first cell type.

According to another embodiment, the method of obtaining a Neural Stem-Like Cell (NSLC), comprises:

- providing a cell of a first type which is not a NSLC;
- increasing intracellular levels of at least one neural stem cell specific polypeptide, wherein the polypeptide is capable of driving directly or indirectly transformation of the cell of the first type into a NSLC; and
- contacting the chromatin and/or DNA of the cell of a first type with a histone acetylator, an inhibitor of histone deacetylation, a DNA demethylator, and/or a chemical inhibitor of DNA methylation.

According to another embodiment, the method of obtaining a Neural Stem-Like Cell (NSLC), comprises:

- obtaining a non-NSLC;
- co-transfecting the non-NSLC with a first polynucleotide encoding a MBD2 polypeptide and with at least one second polynucleotide encoding a MUSASHI1 polypeptide and/or encoding a NGN2 polypeptide;
- placing the co-transfected cell in culture conditions for supporting the transformation of NSLC until said NSLC is obtained.

Certain aspects of the invention concerns isolated cells, cell lines, compositions, 3D assembly of cells, and tissues comprising cells obtained using the methods described herein. Additional aspects concerns the use of such isolated cells, cell lines, compositions, 3D assembly of cells, and tissues of medical treatment and methods of regenerating a mammalian tissue or organ.

Yet, a further aspect concerns a method for repairing or regenerating a tissue in a subject. In one embodiment the method comprises the administration of a reprogrammed cell as defined herein to a subject in need thereof, wherein the administration provides a dose of reprogrammed cells sufficient to increase or support a biological function of a given tissue or organ, thereby ameliorating the subject's condition.

The benefits of the present invention are significant and include lower cost of cell therapy by eliminating the need of immuno-suppressive agents, no need for embryos or fetal tissue, thus eliminating ethical and time constraints, lower cost of production, and no health risks due to possible transmission of viruses or other disease. In addition, since the cells are created fresh, they tend to be more potent than cells that have been passaged multiple times.

Additional aspects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments which are exemplary and should not be interpreted as limiting the scope of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for cell dedifferentiation and cell reprogramming. A significant aspect of the present invention is that it permits the use of a patient's own cells to develop different types of cells that can be transplanted after steps of in vitro dedifferentiation and in vitro reprogramming. Thus, this technology eliminates the problems associated with transplantation of non-host cells, such as, immunological rejection and the risk of transmitting disease. In addition, since the cells are "newly created", they have the potential to be more potent than alternative sources of natural cells that have already divided multiple times.

DEFINITIONS

As used herein and in the appended claims, the singular forms "a," "an", and "the", include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a cell" includes one or more of such cells or a cell line derived from such a cell, reference to "an agent" includes one or more of such agent, and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

As used herein, the term "polynucleotide" refers to any DNA or RNA sequence or molecule, comprising encoding nucleotide sequences. The term is intended to encompass all polynucleotides whether occurring naturally or non-naturally in a particular cell, tissue or organism. This includes DNA and fragments thereof, RNA and fragments thereof, cDNAs and fragments thereof, expressed sequence tags, artificial sequences including randomized artificial sequences.

As used herein, the term "polypeptide" refers to any amino acid sequence having a desired functional biological activity (e.g. DNA demethylation). The term is intended to encompass complete proteins, fragments thereof, fusion proteins and the like, including carbohydrate or lipid chains or compositions.

"Trans-differentiation" refers to a direct switch of an already differentiated cell to another type of differentiated cell.

"De-differentiation" refers to the loss of phenotypic characteristics of a differentiated cell by activating or deactivating genes or metabolic pathways.

"Marker" refers to a gene, polypeptide, or biological function that is characteristic of a particular cell type or cellular phenotype.

"Genetically-engineered DNA sequence" is meant a DNA sequence wherein the component sequence elements of DNA sequence are organized within the DNA sequence in a manner not found in nature.

"Signal sequence" refers to a nucleic acid sequence which, when incorporated into a nucleic acid sequence encoding a polypeptide, directs secretion of the translated polypeptide from cells which express said polypeptide, or allows the polypeptide to readily cross the cell membrane into a cell. The signal sequence is preferably located at the 5' end of the nucleic acid sequence encoding the polypeptide, such that the polypeptide sequence encoded by the signal sequence is located at the N-terminus of the translated polypeptide. By "signal peptide" is meant the peptide sequence resulting from translation of a signal sequence.

"Ubiquitous promoter" refers to a promoter that drives expression of a polypeptide or peptides encoded by nucleic acid sequences to which promoter is operably linked. Preferred ubiquitous promoters include human cytomegalovirus immediate early (CMV); simian virus 40 early promter (SV40); Rous sarcoma virus (RSV); or adenovirus major late promoter.

"Gene expression profiling" means an assay that measures the activity of multiple genes at once, creating a global picture of cellular function. For example, these profiles can distinguish between human neural stem cells and somatic cells that are actively dividing or differentiating.

"Transfection" refers to a method of gene delivery that introduces a foreign nucleotide sequences (e.g. DNA molecules) into a cell preferably by a non-viral method. In preferred embodiments according to the present invention foreign DNA is introduced to a cell by transient transfection of an expression vector encoding a polypeptide of interest, whereby the foreign DNA is introduced but eliminated over time by the cell and during mitosis. By "transient transfection" is meant a method where the introduced expression vectors and the polypeptide encoded by the vector, are not permanently integrated into the genome of the host cell, or anywhere in the cell, and therefore may be eliminated from the host cell or its progeny over time. Proteins, polypeptides, or other compounds can also be delivered into a cell using transfection methods.

"Neuroprogenitor Cell" refers to an immature cell of the nervous system, which can differentiate into neurons and glia (oligodendrocytes and astrocytes). "Neural Stem Cell" is an ectoderm germ layer derived multipotent stem cell having, as a physiological feature, a capacity to form neuroprogenitor cells and under physiological conditions that favor differentiation to form neurons and glia, "Neural Stem-Like Cell" or "NSLC" refers to any cell-derived multipotent stem cell having, as a physiological feature, a capacity to form other neural stem-like cells and neuroprogenitor-like cells and under physiological conditions that favor differentiation to form neuron-like cells and glial-like cells.

"Neurosphere" refers to a cellular aggregate of neural stem cells and neuroprogenitor cells that form a floating sphere formed as a result of proliferation of the neural stem cells and neuroprogenitor cells in appropriate proliferation conditions. NSLCs also form neurospheres consisting of aggregates of NSLCs and neuroprogenitor-like cells.

"Reprogrammed cell" refers to a cell that has undergone stable trans-differentiation, de-differentiation, or transformation. Some reprogrammed cells can be subsequently induced to re-differentiate. The reprogrammed cell stably expresses a cell-specific marker or set of markers, morphology, and/or biological function that was not characteristic of the original cell. "Reprogrammed somatic cell" refers to a process that alters or reverses the differentiation status of a somatic cell, which can be either complete or partial conversion of the differentiated state to an either less differentiated state or a new differentiated state.

"Regeneration" refers to the capability of contributing to the repair or de novo construction of a cell, tissue or organ.

"Differentiation" refers to the developmental process of lineage commitment of a cell. Differentiation can be assayed by measuring an increase in one or more cell-differentiation specific markers relative to the expression of the undifferentiated cell markers.

"Lineage" refers to a pathway of cellular development, in which a more undifferentiated cell undergoes progressive physiological changes to become a more differentiated cell type having a characteristic function (e.g., neurons and glia are of a neuroprogenitor lineage, which is of an ectoderm lineage which formed from blastocysts and embryonic stem (ES) cells).

"Tissue" refers to an ensemble of cells (identical or not) and an extracellular matrix (ECM) that together carry out a specific function or set of functions.

"CDM" is meant a living tissue equivalent or matrix, a living scaffold, or cell-derived matrix.

Cell Transformation

Some aspects of the invention concerns methods and cells to transform or reprogram a given somatic cell into a pluripotent, multipotent and/or unipotent cell. Some aspects of the invention relates to methods for conditioning a somatic cell to reprogramming into a pluripotent, multipotent or unipotent cell.

The terms "transform" or "reprogram" are used interchangeably to refer to the phenomenon in which a cell is dedifferentiated or transdifferentiated to become pluripotent, multipotent and/or unipotent. The dedifferentiated cell could subsequently be redifferentiated into a different type of cell. Cells can be reprogrammed or converted to varying degrees. For example, it is possible that only a small portion of cells are converted or that an individual cell is reprogrammed to be multipotent but not necessarily pluripotent. Thus, the terms "transforming" or "reprogramming" methods can refer to methods wherein it is possible to reprogram a cell such that the "new" cell shows morphological and functional characteristics of a new or different specific cell lineage (e.g. the transformation of fibroblast cells into neuronal cells).

As used herein, the term "somatic cell" refers to any differentiated cell forming the body of an organism, apart from stem cells, progenitor cells, and germline cells (i.e. ovogonies and spermatogonies) and the cells derived therefrom (e.g. oocyte, spermatozoa). For instance, internal organs, skin, bones, blood, and connective tissue are all made up of somatic cells. Somatic cells according to the invention can be differentiated cells isolated from adult or can be fetal somatic cells. Somatic cells are obtained from animals, preferably human subjects, and cultured according to standard cell culture protocols available to those of ordinary skill in the art.

As used herein, "Stem cell" refers to those cells which retain the ability to renew themselves through mitotic cell division and which can differentiate into a diverse range of specialized cell types. It includes both embryonic stem cells that are found in blastocysts, and adult stem cells that are found in adult tissues. "Totipotent cells" refers to cells that have the ability to develop into cells derived from all three embryonic germ layers (mesoderm, endoderm and ectoderm) and an entire organism (e.g., human being if placed in a woman's uterus in the case of humans). Totipotent cells may give rise to an embryo, the extra embryonic membranes and all post-embryonic tissues and organs. The term "pluripotent" as used herein is intended to mean the ability of a cell to give rise to differentiated cells of all three embryonic germ layers. "Multipotent cells" refers to cells that can produce only cells of a closely related family of cells (e.g. hematopoietic stem cells differentiate into red blood cells, white blood cells, platelets, etc.). "Unipotent cells" refers to cells that have the capacity to develop/differentiate into only one type of tissue/cell type (e.g. skin cells).

The present invention allows the reprogramming of any cell to a different type of cell. Although the present application focuses primarily on the preparation of Stem-Like cells, especially, Neural Stem-Like Cells (NSLCs), the invention is not so restricted because many different types of cells can be generated according to the principles described herein. Similarly, while the Examples section describes embodiments where fibroblasts, keratinocytes, $CD34^+$ cells, adipose-derived stem cells (ADSCs), neural stem cells (including NSLCs), and cells within a Cell-Derived Matrix (CDM) are reprogrammed, the invention is not limited such cells. The invention may be employed for the reprogramming of virtually any cell of interest.

Accordingly, a general aspect of the invention relates to a method of transforming a cell of a first type to a cell of a second different type. As used herein, examples of cells of a first type include, but are not limited to germ cells, embryonic stem cells and derivations thereof, adult stem cells and derivations thereof, progenitor cells and derivations thereof; cells derived from mesoderm, endoderm or ectoderm, and a cell of mesoderm, endoderm or ectoderm lineage such as an adipose-derived stem cell (ADSC), mesenchymal stem cell, hematopoietic stem cell ($CD34^+$ cell), skin derived precursor cell, hair follicle cell, fibroblast, keratinocyte, epidermal cell, endothelial cell, epithelial cell, granulosa epithelial cell, melanocyte, adipocyte, chondrocyte, hepatocyte, lymphocyte (B and T lymphocyte), granulocyte, macrophage, monocyte, mononuclear cell, pancreatic islet cell, sertoli cell, neuron, glial cell, cardiac muscle cell, and other muscle cell.

As used herein, examples of cells of a second type include, but are not limited to germ cells, embryonic stem cells and derivations thereof, adult stem cells and derivations thereof, progenitor cells and derivations thereof, cells derived from mesoderm, endoderm or ectoderm, and a cell of mesoderm, endoderm or ectoderm lineage such as an adipose-derived stem cell, mesenchymal stem cell, hematopoietic stem cell, skin derived precursor cell, hair follicle cell, fibroblast, keratinocyte, epidermal cell, endothelial cell, epithelial cell, granulosa epithelial cell, melanocyte, adipocyte, chondrocyte, hepatocyte, lymphocyte (B and T lymphocyte), granulocyte, macrophage, monocyte, mononuclear cell, pancreatic islet cell, sertoli cell, neuron, glial cell, cardiac muscle cell, and other muscle cell. In addition, each of the above "-like" cell (a cell that has similar but not completely identical characteristics of the known natural type of the cell) is also included in the examples of cells of a second type.

According to one particular aspect, the method of transforming a cell of a first type into a cell of a second different type comprises the steps of:

i) providing a cell of a first type;
ii) transiently increasing in the cell of a first type intracellular levels of at least one reprogramming agent, whereby the transient increase induces direct or indirect endogenous expression of at least one gene regulator;
iii) placing the cell in conditions for supporting the transformation of the desired cell and maintaining intracellular levels of the at least one reprogramming agent for a sufficient period of time to allow stable expression of the at least one gene regulator in absence of the reprogramming agent; and
iv) maintaining the cell in culture conditions supporting the transformation of the desired cell for a sufficient period of time to allow a stable expression of a plurality of secondary genes whose expression is characteristic of phenotypical and functional properties of the desired cell. At least one of the stably expressed secondary genes is not characteristic of phenotypical and functional properties of an embryonic stem cell. At the end of said period of time the cell of the first type has been transformed into the desired cell of a different type. Preferably, the cell of a different type obtained after the transformation is further characterized by a stable repression of a plurality of genes expressed in the first cell type.

According to various embodiments, step iii) may be carried out consecutively to step ii), simultaneously with step ii), or before step ii).

According to a related aspect, the invention relates to a process wherein a cell of a first type is reprogrammed to a desired cell of a different type, the process comprising:

a transient increase of intracellular levels of at least one reprogramming agent, wherein the at least one reprogramming agent induces a direct or indirect endogenous expression of at least one gene regulator, wherein the endogenous expression of the at least one gene regulator is necessary for the existence of the desired cell of a different type;

a stable expression of said at least one gene regulator;

stable expression of a plurality of secondary genes, wherein the stable expression of the plurality of secondary genes is the result of the stable expression of the at least one gene regulator, and wherein: (i) stable expression of the plurality of secondary genes is characteristic of phenotypical and/or functional properties of the desired cell, (ii) stable expression of at least one of the secondary genes is not characteristic of phenotypical and functional properties of an embryonic stem cell, and wherein (i) and (ii) are indicative of successful reprogramming of the cell of the first type to the desired cell of the different type.

As used herein, "transiently increasing" refers to an increase that is not necessarily permanent and therefore, which may decrease or disappear over time. For instance, when referring to transiently increasing intracellular levels of at least one reprogramming agent in a cell, it means that the increase in present for a sufficient period of time for causing particular cellular events to occur (e.g. inducing stable endogenous expression of a gene regulator). Typically a transient increase is not permanent and is not associated for instance to genome integration of an expression vector.

As used herein the term "reprogramming agent" refers to a compound that is capable of inducing directly or indirectly the expression of morphological and/or functional characteristics of the desired cell of a different type. Preferred compounds include those capable of driving directly or indirectly transformation of the cell of the first type into the desired cell of a different type. In preferred embodiment, the reprogramming agent is selected for inducing a direct or indirect endogenous expression of at least one gene regulator as defined herein. There are many compounds that may be helpful in reprogramming a cell according to the invention and these compounds can be used alone or in combinations. In various embodiments, the reprogramming agent is a polynucleotide or polypeptide selected according to TABLE A:

TABLE A

Reprogramming agent					
Examples of Desired Cell Type	Name	RefSeq/ GenBank ™ (NCBI) Access. No.	UniProt ™/ Swiss-Prot Access. No.	UniGene ™ Accession No.	Markers
Pluripotent-like cells	AGR2	NM_006408.3	O95994	Hs.530009	OCT4
	AGR3	NM_176813.3	Q8TD06	Hs.100686	Nanog
	BRIX1	NM_018321.3	Q8TDN6	Hs.718510	SSEA-4
	CRABP2	NM_001878.2	P29373	Hs.405662	TRA1-60
	DNMT3B, isoform 1	NM_006892.3	Q9UBC3	Hs.713611	TRA1-80
	DNMT3B, isoform 2	NM_175848.1	Q9UBC3	Hs.713611	AP
	DNMT3B, isoform 3	NM_175849.1	Q9UBC3	Hs.713611	
	DNMT3B, isoform 6	NM_175850.1	Q9UBC3	Hs.713611	
	DPPA2	NM_138815.3	Q7Z7J5	Hs.351113	
	DPPA3 (STELLA)	NM_199286.2	Q6W0C5	Hs.131358	
	DPPA4	NM_018189.3	Q7L190	Hs.317659	
	DPPA5 (ESG1)	NM_001025290.1	A6NC42	Hs.125331	
	FOXD3	NM_012183.2	Q9UJU5	Hs.546573	
	FOXH1	NM_003923.2	O75593	Hs.708365	
	GABRB3, isoform 1	NM_000814.5	P28472	Hs.302352	
	GABRB3, isoform 2	NM_021912.4	P28472	Hs.302352	
	GABRB3, isoform 3	NM_001191320.1	P28472	Hs.302352	
	GABRB3, isoform 4	NM_001191321.1	P28472	Hs.302352	
	GBX2	NM_001485.2	P52951	Hs.184945	
	GDF3	NM_020634.1	Q9NR23	Hs.86232	
	GJA1 (CX43)	NM_000165.3	P17302	Hs.74471	
	GRB7	NM_005310.2	Q14451	Hs.86859	
		NM_001030002.1	Q14451	Hs.86859	
	HESRG	NR_027122.1	Q1W209	Hs.720658	
	IFITM1	NM_003641.3	P13164	Hs.458414	
	IFITM2	NM_006435.2	Q01629	Hs.709321	
	KLF2	NM_016270.2	Q9Y5W3	Hs.726356	
	KLF4	NM_004235.4	O43474	Hs.376206	
	LEFTY1	NM_020997.2	O75610	Hs.656214	
	LEFTY2 (EBAF), isoform 1	NM_003240.3	O00292	Hs.520187	
	LEFTY2 (EBAF), isoform 2	NM_001172425.1	B4E332 (TrEMBL)	Hs.520187	
	LIN28A	NM_024674.4	Q9H9Z2	Hs.86154	
	MYBL2	NM_002466.2	P10244	Hs.179718	
	NANOG	NM_024865.2	Q9H9S0	Hs.635882	
	NODAL	NM_018055.4	Q96S42	Hs.370414	
	NOG	NM_005450.4	Q13253	Hs.248201	
	NR0B1 (DAX1)	NM_000475.4	P51843	Hs.268490	
	NR5A2, isoform 1	NM_205860.1	O00482	Hs.33446	
	NR5A2, isoform 2	NM_003822.3	O00482	Hs.33446	
	NR6A1, isoform 1	NM_033334.2	Q15406	Hs.586460	
	NR6A1, isoform 2	NM_001489.3	Q15406	Hs.586460	
	PHC1	NM_004426.2	P78364	Hs.305985	
	PITX2, isoform a	NM_153427.1	Q99697	Hs.643588	
	PITX2, isoform b	NM_153426.1	Q99697	Hs.643588	
	PITX2, isoform c	NM_000325.5	Q99697	Hs.643588	
	PODXL, isoform 1	NM_001018111.2	O00592	Hs.726449	
	PODXL, isoform 2	NM_005397.3	O00592	Hs.726449	
	POU5F1 (OCT4), isoform 1*	NM_002701.4	O01860	Hs.249184	

TABLE A-continued

Examples of Desired Cell Type	Name	RefSeq/ GenBank ™ (NCBI) Access. No.	UniProt ™/ Swiss-Prot Access. No.	UniGene ™ Accession No.	Markers
	Reprogramming agent				
	POU5F1 (OCT4), isoform 2	NM_203289.4 NM_001173531.1	N/A	Hs.249184	
	PTEN	NM_000314.4	P60484	Hs.500466	
	REST	NM_005612.4	Q13127	Hs.307836	
		NM_001193508.1	Q13127	Hs.307836	
	REX1	NM_020695.3	Q8N1G1	Hs.192477	
	SALL4	NM_020436.3	Q9UJQ4	Hs.517113	
	SEMA3A	NM_006080.2	Q14563	Hs.252451	
	SFRP2	NM_003013.2	Q96HF1	Hs.481022	
	SOX2	NM_003106.2	P48431	Hs.518438	
	TDGF1, isoform 1	NM_003212.3	P13385	Hs.385870	
	TDGF1, isoform 2	NM_001174136.1	P13385	Hs.385870	
	TERT, isoform 1	NM_198253.2	O14746	Hs.492203	
	TERT, isoform 2	NM_001193376.1	O14746	Hs.492203	
	TPT1	NM_003295.2	P13693	Hs.374596	
	UTF1	NM_003577.2	Q5T230	Hs.458406	
	ZFP42	NM_174900.3	Q96MM3	Hs.335787	
Ectoderm-like cells	ASCL1 (MASH1)	NM_004316.3	P50553	Hs.703025	FoxJ3
	CDX1	NM_001804.2	P47902	Hs.1545	Otx2
	DLX3	NM_005220.2	O60479	Hs.134194	E-cadherin
	DLX5	NM_005221.5	P56178	Hs.99348	TP73L
	FOXD3	NM_012183.2	Q9UJU5	Hs.546573	
	MSI1	NM_002442.2	O43347	Hs.158311	
	NANOG	NM_024865.2	Q9H9S0	Hs.635882	
	POU5F1 (OCT4), isoform 1*	NM_002701.4	Q01860	Hs.249184	
	POU5F1 (OCT4), isoform 2	NM_203289.4 NM_001173531.1	N/A	Hs.249184	
	SOX1	NM_005986.2	O00570	Hs.202526	
	SOX2	NM_003106.2	P48431	Hs.518438	
	SP8, isoform 1	NM_182700.4	Q8IXZ3	Hs.195922	
	SP8, isoform 2	NM_198956.2	N/A	Hs.195922	
	ZIC1	NM_003412.3	Q15915	Hs.647962	
Mesendoderm-like cells	EOMES	NM_005442.2	O95936	Hs.591663	Mixl1
	FOXA2, isoform 1*	NM_021784.4	Q9Y261	Hs.155651	Mesp1
	FOXA2, isoform 2	NM_153675.2	Q9Y261	Hs.155651	Bry
	FOXD3	NM_012183.2	Q9UJU5	Hs.546573	Flk1
	GATA4	NM_002052.3	P43694	Hs.243987	Pax2
	GATA6	NM_005257.3	Q92908	Hs.514746	Six1
	MIXL1	NM_031944.1	Q9H2W2	Hs.282079	
	POU5F1 (OCT4), isoform 1*	NM_002701.4	Q01860	Hs.249184	
	POU5F1 (OCT4), isoform 2	NM_203289.4 NM_001173531.1	N/A	Hs.249184	
	SOX17	NM_022454.3	Q9H6I2	Hs.98367	
	T (Brachyury)	NM_003181.2	O15178	Hs.389457	
Desired second cell type					
Neural stem-like cells	CALB1	NM_004929.2	P05937	Hs.65425	Sox2
	DLL1	NM_005618.3	O00548	Hs.379912	Nestin
	DLX1, isoform 1	NM_178120.4	P56177	Hs.407015	GFAP
	DLX1, isoform 2	NM_001038493.1	P56177	Hs.407015	Msi1
	DLX2	NM_004405.3	Q07687	Hs.419	Sox1
	FOXD3	NM_012183.2	Q9UJU5	Hs.546573	CD133
	GJD2 (CX36)	NM_020660.1	Q9UKL4	Hs.283816	
	HES1	NM_005524.2	Q14469	Hs.250666	
	HES3	NM_001024598.3	Q5TGS1	Hs.532677	
	HES5	NM_001010926.3	Q5TA89	Hs.57971	
	HOXB1	NM_002144.3	P14653	Hs.99992	
	MNX1 (HB9), isoform 1	NM_005515.3	P50219	Hs.37035	
	MNX1 (HB9), isoform 2	NM_001165255.1	N/A	Hs.37035	
	MSI1	NM_002442.2	O43347	Hs.158311	
	NANOG	NM_024865.2	Q9H9S0	Hs.635882	
	NEUROD1	NM_002500.2	Q13562	Hs.709709	
	NEUROG1	NM_006161.2	Q92886	Hs.248149	
	NEUROG2	NM_024019.2	Q9H2A3	Hs.567563	
	NKX6.1	NM_006168.2	P78426	Hs.546270	
	PAX6, isoform a*	NM_000280.3	P26367	Hs.270303	

TABLE A-continued

Reprogramming agent					
Examples of Desired Cell Type	Name	RefSeq/ GenBank ™ (NCBI) Access. No.	UniProt ™/ Swiss-Prot Access. No.	UniGene ™ Accession No.	Markers
	PAX6, isoform a	NM_001127612.1	P26367	Hs.270303	
	PAX6, isoform b	NM_001604.4	P26367	Hs.270303	
	SFRP2	NM_003013.2	Q96HF1	Hs.481022	
	SIX3	NM_005413.3	O95343	Hs.567336	
	SOX1	NM_005986.2	O00570	Hs.202526	
	SOX2	NM_003106.2	P48431	Hs.518438	
Cardiac progenitor-like cells	BAF60C (SMARCD3), isoform 1	NM_001003802.1	Q6STE5	Hs.647067	MLc2α
	BAF60C (SMARCD3), isoform 1	NM_003078.3	Q6STE5	Hs.647067	Nkx2.5
	BAF60C (SMARCD3), isoform 2*	NM_001003801.1	Q6STE5	Hs.647067	Isl+
	FOXD3	NM_012183.2	Q9UJU5	Hs.546573	Bry
	GATA4	NM_002052.3	P43694	Hs.243987	
	GATA6	NM_005257.3	Q92908	Hs.514746	
	HAND1	NM_004821.2	O96004	Hs.152531	
	HAND2	NM_021973.2	P61296	Hs.388245	
	ISL1	NM_002202.2	P61371	Hs.505	
	KDR	NM_002253.2	P35968	Hs.479756	
	MESP1	NM_018670.3	Q9BRJ9	Hs.447531	
	MYOCD, isoform 1	NM_001146312.1	Q6N065 (TrEMBL)	Hs.567641	
	MYOCD, isoform 2	NM_153604.2	Q8IZQ8	Hs.567641	
	MYOCD, isoform 3	NM_001146313.1	Q8IZQ8	Hs.567641	
	NKX2.5, isoform 1*	NM_004387.3	P52952	Hs.54473	
	NKX2.5, isoform 2*	NM_001166175.1	P52952	Hs.54473	
	NKX2.5, isoform 3*	NM_001166176.1	P52952	Hs.54473	
	T (Brachyury)	NM_003181.2	O15178	Hs.389457	
	TBX5, isoform 1*	NM_000192.3	Q99593	Hs.381715	
	TBX5, isoform 1	NM_181486.1	Q99593	Hs.381715	
	TBX5, isoform 2	NM_080718.1	Q99593	Hs.381715	
	TBX5, isoform 3	NM_080717.2	Q99593	Hs.381715	
	SOX17	NM_022454.3	Q9H6I2	Hs.98367	
Pancreatic progenitor-like cells	FOXA2, isoform 1*	NM_021784.4	Q9Y261	Hs.155651	PDX1
	FOXA2, isoform 2	NM_153675.2	Q9Y261	Hs.155651	Sox17
	FOXD3	NM_012183.2	Q9UJU5	Hs.546573	FoxA2
	MAFA	NM_201589.2	Q8NHW3	Hs.670866	Ngn3
	MIXL1	NM_031944.1	Q9H2W2	Hs.282079	Isl1
	NEUROG3	NM_020999.3	Q9Y4Z2	Hs.532682	
	NKX6.1	NM_006168.2	P78426	Hs.546270	
	PAX4	NM_006193.2	O43316	Hs.129706	
	PDX1	NM_000209.3	P52945	Hs.32938	
	SOX17	NM_022454.3	Q9H6I2	Hs.98367	
Myogenic progenitor-like cells	FOXC1	NM_001453.2	Q12948	Hs.348883	SMα actin
	FOXC2	NM_005251.2	Q99958	Hs.436448	Calponin
	MEF2C, isoform 1	NM_002397.4 NM_001193350.1	Q06413 Q06413	Hs.649965	MyoD
	MEF2C, isoform 2	NM_001131005.2	Q06413	Hs.649965	MEF2C
	MEF2C, isoform 3	NM_001193347.1	Q06413	Hs.649965	Pax3
	MEF2C, isoform 4	NM_001193348.1	Q06413	Hs.649965	Pax7
	MEF2C, isoform 5	NM_001193349.1	Q06413	Hs.649965	
	Pax3, isoform Pax3	NM_181457.3	P23760	Hs.42146	
	Pax3, isoform Pax3a	NM_000438.5	P23760	Hs.42146	
	Pax3, isoform Pax3b	NM_013942.4	P23760	Hs.42146	
	Pax3, isoform Pax3d	NM_181458.3	Q494Z3, Q494Z4 (TrEMBL)	Hs.42146	
	Pax3, isoform Pax3e	NM_181459.3	Q494Z3, Q494Z4 (TrEMBL)	Hs.42146	
	Pax3, isoform Pax3g	NM_181461.3	Q494Z3, Q494Z4 (TrEMBL)	Hs.42146	
	Pax3, isoform Pax3h	NM_181460.3	Q494Z3, Q494Z4 (TrEMBL)	Hs.42146	
	Pax3, isoform Pax3i	NM_001127366.2	Q494Z4 (TrEMBL)	Hs.42146	
	PAX7, isoform 1	NM_002584.2	P23759	Hs.113253	
	PAX7, isoform 2	NM_013945.2	P23759	Hs.113253	
	PAX7, isoform 3	NM_001135254.1	P23759	Hs.113253	

In some embodiments, the reprogramming agent is a polypeptide which shares at least 75%, 80%, 85%, 90%, 95%, 97%, 99% or more of the functionality or sequence identity of any one of the reprogramming agents in the table hereinbefore.

Identifying the "sufficient period of time" to allow stable expression of the at least one gene regulator in absence of the reprogramming agent and the "sufficient period of time" in which the cell is to be maintained in culture conditions supporting the transformation of the desired cell is within the skill of those in the art. The sufficient or proper time period will vary according to various factors, including but not limited to, the particular type and epigenetic status of cells (e.g. the cell of the first type and the desired cell), the amount of starting material (e.g. the number of cells to be transformed), the amount and type of reprogramming agent(s), the gene regulator(s), the culture conditions, presence of compounds that speed up reprogramming (ex, compounds that increase cell cycle turnover, modify the epigenetic status, and/or enhance cell viability), etc. In various embodiments the sufficient period of time to allow a stable expression of the at least one gene regulator in absence of the reprogramming agent is about 1 day, about 2-4 days, about 4-7 days, about 1-2 weeks, about 2-3 weeks or about 3-4 weeks. In various embodiments the sufficient period of time in which the cells are to be maintained in culture conditions supporting the transformation of the desired cell and allow a stable expression of a plurality of secondary genes is about 1 day, about 2-4 days, about 4-7 days, or about 1-2 weeks, about 2-3 weeks, about 3-4 weeks, about 4-6 weeks or about 6-8 weeks. In preferred embodiments, at the end of the transformation period, the number of transformed desired cells is substantially equivalent or even higher than an amount of cells a first type provided at the beginning.

The present invention encompasses various types of compounds that are suitable for increasing in a cell of a first type the intracellular levels of at least one reprogramming agent. Preferably, the compound should also be able to directly or indirectly remodel the chromatin and/or DNA of the cell, thus resulting directly or indirectly in the expression of morphological and functional characteristics of the desired cell of a different type. Preferred compounds are reprogramming agents as defined herein or any other compound having a similar activity and having the ability to activate or enhance the expression of the endogenous version of genes listed in the table of reprogramming agents hereinbefore and which are capable of driving directly or indirectly transformation of the cell of the first type into the desired cell of a different type.

As will be explained hereinafter, the increase in intracellular levels of the at least one reprogramming agent can be achieved by different means. In preferred embodiments the reprogramming agent is a polypeptide and increasing intracellular levels of such polypeptide include transfection (or co-transfection) of an expression vector having a polynucleotide (ex. DNA or RNA) encoding the polypeptide(s), or by an intracellular delivery of polypeptide(s). According to the invention, transient expression is generally preferable. Additional suitable compounds may include compounds capable of increasing the expression of the endogenous version of genes listed in the table of reprogramming agents and gene regulators including, but not limited to, reprogramming factors listed in Table B.

TABLE B

Desired cell of different type	Reprogramming Factor
Pluripotent-like cells	Nodal, ActivinA, Fgf-2, Wnt3a, L-Ascorbic Acid, BIO, CHIR99021, PD0325901, Thiazovivin, SB431542, Cyclic Pifithrin-α, Tranylcypromine hydrochloride, Kenpaullone, 5-Azacytidine, Valproic Acid, BIX01294, R(+)BayK8644, RG108, Theanine, Sodium butyrate
Ectoderm like cells: 1—Neural stem-like cells	a retinoid compound, L-Ascorbic acid, SHH, Wnt 3a, a neurotrophic factor, bFGF, EGF, Transforming growth factor alpha, neuropeptide Y, Estrogen, Noggin, Forskolin, 5-Azacytidine, Valproic Acid, BIX01294, R(+)BayK8644, RG108, Sodium butyrate, Lithium
Mesoendoderm like cells:	BMP4, Epidermal growth factor-Cripto/FRL-1/Cryptic (EGF-CFC) and the TGFβs, Activin, Nodal, SHH, Vg1/GDF1 (growth and differentiation factor-1)
1—Cardiac progenitor-like cells	1—BMP4, bFGF, Activin A, VEGF, DKK1 (dickkopf homologue 1), Insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF), 5-Azacytidine, Valproic Acid, BIX01294, R(+)BayK8644, RG108, Cardiogenol C hydrochloride, Sodium butyrate
2—Pancreatic progenitor-like cells	2—Activin A, GLP-1, bFGF, Reg1, nicotinamide, Betacellulin, SHH, (−)-Indolactam V, a retinoid compound, Cyclopamine, IDE-1 and 2, 5-Azacytidine, Valproic Acid, BIX01294, R(+)BayK8644, RG108, Sodium butyrate
3—Myogenic progenitor-like cells	3—retinoic acid, HGF, FGF, IGF, transforming growth factor-beta, Wnt3a, 5-Azacytidine, Valproic Acid, BIX01294, R(+)BayK8644, RG108, Sodium butyrate

According to the principles of the invention, increasing intracellular levels of at least one reprogramming agent should induce a direct or indirect endogenous expression of at least one gene regulator. As used herein, "gene regulator" refers to a polynucleotide or polypeptide whose expression is associated with a series of intracellular events leading to the transformation of a given cell of a first type into a pluripotent, multipotent and/or unipotent cell. Typically expression of a gene regulator directly or indirectly activates genes necessary for the phenotypical and functional characteristic of pluripotent, multipotent and/or unipotent cells, while repressing genes of the cell of a first type. The gene regulator may be the same or be different than the reprogramming agent. Examples of gene regulators according to the invention include, but are not limited to, the polynucleotides and polypeptides listed herein before in TABLE A.

In some embodiments, the gene regulator is a polypeptide which shares at least 75%, 80%, 85%, 90%, 95%, 97%, 99% or more of the functionality or sequence identity of any one of the gene regulators provided in the Table A hereinbefore.

As used herein, "conditions supporting growth" or "conditions supporting the transformation" when referring to a desired cell refers to various suitable culture conditions (temperature, pH, O_2 tension, cell media, factors, compounds, growth substrate (ex. laminin, collagen, fibronectin, Matrigel™, low-bind surface, nanostructured or charged surface, etc.), 3D environment, etc.) favorising growth of the desired cell type and/or favorising transformation towards such desired cell type. Those skilled in the art known that growth or transformation of particular cell types is stimulated under specific conditions, while inhibited by others, and it is within their skill to select suitable conditions (e.g. culture conditions) favorising growth or transformation of desired cell types.

The terms "phenotypical and functional properties", when referring to a desired cell or to an embryonic stem cell, means the biological, biochemical, physiological and visual characteristics of a cell, including expression of certain genes and cell surface markers, which can be measured or assessed for confirming its identity or function(s).

An example of a suitable reprogramming agent according to preferred embodiments of the invention is MUSASHI1. In some embodiments this polypeptide is preferred for driving a first cell, such as a fibroblast, into a Neural Stem-Like Cell (NSLC). In other embodiments, the at least one reprogramming agent which said intracellular levels is increased is(are) either Musashi1 (Msi1) alone; Musashi1 (Msi1) and Neurogenin 2 (Ngn2); Musashi1 (Msi1) and methyl-CpG binding domain protein 2 (MBD2); or Neurogenin 2 (Ngn2) and methyl-CpG binding domain protein 2 (MBD2). Adequate intracellular levels of these polypeptides are preferred since they tend to be expressed throughout an entire cell lineage, from as early as embryonic stem cells (or even earlier) to pre-somatic cells (or even later).

MBD2 is a member of a family of methyl-CpG-binding proteins that has been reported to be both a transcriptional repressor and a DNA demethylase (dMTase). As used herein, the term "MBD2" generally refers to the human methyl-CpG binding domain protein 2. The GeneBank™ (NCBI) accession number of human MBD2 is NM_003927.3/AF072242, the UniProt™ accession number is NP-003918/Q9UBB5 and the UniGene™ accession number is Hs.25674.

As used herein, the term "Msi1" generally refers to the human musashi homolog 1. The GeneBank™ (NCBI) accession number of human Msi1 is NM_002442.2/AB012851, the UniProt™ accession number is NP-002433/043347 and the UniGene™ accession number is Hs.158311.

As used herein, the term "Ngn2" generally refers to the human neurogenin 2. The GeneBank™ (NCBI) accession number of human Ngn2 is NM_024019.2/BC036847, the UniProt™ accession number is NP-076924/Q9H2A3 and the UniGene™ accession number is Hs.567563.

According to additional aspects, the method of transforming a cell of a first type to a desired cell of a different type comprises the steps of either:

1) contacting the cell of a first type with one or more compounds capable of increasing intracellular levels of at least one reprogramming agent within the cell and directly or indirectly remodeling the chromatin and/or DNA of the cell; or
2) contacting the chromatin and/or DNA of a cell of a first type with an agent capable of remodeling the chromatin and/or DNA of the cell; and increasing intracellular levels of at least one reprogramming agent.

According to various embodiments, step 2) may be carried out consecutively to step 1), simultaneously with step 1), or before step 1).

According to a particular aspect, the invention relates to a method for obtaining a Neural Stem-Like Cell (NSLC), comprising:

providing a cell of a first type which is not a NSLC;
increasing intracellular levels of at least one neural stem cell specific polypeptide, wherein the polypeptide is capable of driving directly or indirectly transformation of the cell of the first type into a NSLC; and
contacting chromatin and/or DNA of a cell of a first type with a histone acetylator, an inhibitor of histone deacetylation, a DNA demethylator, and/or a chemical inhibitor of DNA methylation.

With respect to the second step, the term "remodelling the chromatin and/or DNA" refers to dynamic structural changes to the chromatin. These changes can range from local changes necessary for transcriptional regulation, to global changes necessary for opening up the chromatin structure or chromosome segregation to allow transcription of the new set of genes characteristic of the desired cell of a different type, to closing up of the chromatin structure or chromosome segregation to prevent transcription of certain genes that are not characteristic of the desired cell of a different type. In some embodiments, opening up of the chromatin structure refers more specifically to acetylation of histones, and demethylation of DNA, while closing up of the chromatin structure refers more specifically to deacetylation of histones, and methylation of DNA.

As used herein, "compound" refers to a compound capable of effecting a desired biological function. The term includes, but is not limited to, DNA, RNA, protein, polypeptides, and other compounds including growth factors, cytokines, hormones or small molecules. As used herein, compounds capable of remodeling chromatin and/or DNA include, but are not limited to, histone acetylators, inhibitors of histone deacetylation, DNA demethylators, inhibitors of DNA methylation and combination thereof.

"Inhibitor of DNA methylation" refers to an agent that can inhibit DNA methylation. DNA methylation inhibitors have demonstrated the ability to restore suppressed gene expression. Suitable agents for inhibiting DNA methylation include, but are not limited to 5-azacytidine, 5-aza-2-deoxycytidine, 1-β-D-arabinofuranosil-5-azacytosine, and dihydro-5-azacytidine, and zebularine (ZEB), BIX (histone lysine methytransferase inhibitor), and RG108.

"Inhibitor of histone deacetylation" refers to an agent that prevents the removal of the acetyl groups from the lysine residues of histones that would otherwise lead to the formation of a condensed and transcriptionally silenced chromatin. Histone deacetylase inhibitors fall into several groups, incuding: (1) hydroxamic acids such as trichostatin (A), (2) cyclic tetrapeptides, (3) benzamides, (4) electrophilic ketones, and (5) aliphatic acid group of compounds such as phenylbutyrate and valporic acid. Suitable agents to inhibit histone deacetylation include, but are not limited to, valporic acid (VPA), phenylbutyrate Trichostatin A (TSA), Na-butyrate, and benzamides. VPA promotes neuronal fate and inhibits glial fate simultaneously through the induction of neurogenic transcription factors including NeuroD.

"Histone Acetylator" refers to an agent that inserts acetyl groups to the lysine residues of histones that opens up the chromatin and turns it into a transcriptionally active state. Suitable Histone Acetylator agents include, but are not limited to, Polyamine, CREB (cAMP element binding protein), and BniP3.

"DNA demethylator" refers to an agent that removes the methyl groups from DNA and possesses the ability to inhibit hypermethylation and restore suppressed gene expression. A demethylase is expected to activate genes by removing the repressive methyl residues. Suitable DNA demethylators include, but are not limited to, MBD2 and Gadd45b.

In some embodiments, the reprogramming agent has one or more of the following functions: it decrease the expression of one or more markers of cells of the first type (ex. see Table C), and/or increase the expression of one or more markers of the desired cell of the different type (ex. see Table A). Cells that exhibit a selectable marker for the desired cell of a different type are then selected and assessed for characteristics of the desired cell of a different type.

According to the invention, transformation into the desired cell results in stable expression of a plurality of secondary genes whose expression is characteristic of phenotypical and/or functional properties of the desired cell. Genes whose expression is characteristic of phenotypical and/or functional properties of the desired cell include, but is not limited to, those listed in Table A.

In some embodiments, expression of secondary genes whose expression is characteristic of phenotypical and functional properties of the desired cell results in the expression of markers defined according to the following table:

Desired cell type	Markers
Neural stem-like cells	Nestin, Sox2, GFAP, Msi1
Neural-like cells	βIII-tubulin, Map2b, Synapsin, ACHE
Ectoderm-like cells	Sox2, Sox1, Zic1, Nestin, Notch 1, FoxJ3, Otx2, Cripto1, Vimentin
Mesendoderm-like cells	Sox17, FoxA2, CXCR4, GATA4, Mixl1, Eomesodermin
Pluripotent-like cells	Oct4, SSEA4, TRA-1-60, TRA-1-81, AP

In some embodiments, transformation of a cell of a first type into the desired cell results in a stable repression of a plurality of genes typically expressed in the cell of the first type. Examples of such suppressed genes include, but are not limited to, those defined in Table C:

TABLE C

Examples of suppressed genes

Cell Type	Cell-type specific genes typically repressed during Reprogramming: Name	RefSeq/ GenBank ™ (NCBI) Accession No.	UniProt ™/ Swiss-Prot Accession No.	UniGene ™ Accession No.	Markers
Keratinocytes	TP63, isoform 1	NM_003722.4	Q9H3D4	Hs.137569	Keratin 14
	TP63, isoform 2	NM_001114978.1	Q9H3D4	Hs.137569	Basonuclin
	TP63, isoform 3	NM_001114979.1	Q9H3D4	Hs.137569	P63
	TP63, isoform 4	NM_001114980.1	Q9H3D4	Hs.137569	
	TP63, isoform 5	NM_001114981.1	Q9H3D4	Hs.137569	
	TP63, isoform 6	NM_001114982.1	Q9H3D4	Hs.137569	
	BNC1	NM_001717.3	Q01954	Hs.459153	
	BCN2	NM_017637.5	Q6ZN30	Hs.656581	
	KRT14	NM_000526.4	P02533	Hs.654380	
	Involucrin	NM_005547.2	P07476	Hs.516439	
Fibroblasts	THY1	NM_006288.3	P04216	Hs.724411	Col5A2
	FBN2	NM_001999.3	P35556	Hs.519294	Fibronectin
	COL5A2	NM_000393.3	P05997	Hs.445827	
	DNMT1, isoform a	NM_001130823.1	P26358	Hs.202672	
	DNMT1, isoform b	NM_001379.2	P26358	Hs.202672	
CD34+	Isl1	NM_002202.2	P61371	Hs.505	VEGFR
	HOXA9	NM_152739.3	P31269	Hs.659350	Cytokeratin
	HOXB4	NM_024015.4	P17483	Hs.664706	
	Klk-1	NM_002257.2	P06870	Hs.123107	CD34
	Bry	NM_003181.2	O15178	Hs.389457	
Adipose-derived stem cells (ADSC)	ALCAM	NM_001627.2	Q13740	Hs.591293	ALBO
	VCAM-1	NM_001078.2	P19320	Hs.109225	Adiponectin
	VCAM-1, isoform b	NM_080682.1	P19320	Hs.109225	
	PROM1, isoform 1	NM_006017.2	O43490	Hs.614734	Leptin
	PROM1, isoform 2	NM_001145847.1 NM_001145848.1	O43490	Hs.614734	
	PROM1, isoform 4	NM_001145852.1	O43490	Hs.614734	
	PROM1, isoform 5	NM_001145851.1	O43490	Hs.614734	
	PROM1, isoform 6	NM_001145850.1	O43490	Hs.614734	
	PROM1, isoform 7	NM_001145849.1	O43490	Hs.614734	
	FUT4	NM_002033.3	P22083	Hs.390420	

In preferred embodiments, stable repression of any one or more of the genes listed in Table C being expressed in the first cell type is also characterized by a disappearance of the corresponding markers (see Table C).

Those skilled in the art will understand that there, exist many alternative steps for facilitating cell reprogramming. Those include destabilizing the cell's cytoskeletal structure (for example, by exposing the cell to cytochalasin B), loosening the chromatin structure of the cell (for example, by using agents such as 5 azacytidine (5-Aza) and Valproic acid (VPA) or DNA demethylator agents such as MBD2), transfecting the cell with one or more expression vector(s) containing at least one cDNA encoding a neurogenic transcription factor (for example, Msi1 or Ngn2), using an appropriate medium for the desired cell of a different type and an appropriate differentiation medium to induce differentiation commitment of the desired cell of a different type, inhibiting repressive pathways that negatively affects induction into commitment the desired cell of a different type, growing the cells on an appropriate substrate for the desired cell of a different type (for example, laminin for NSLCs or a low-bind surface for culturing floating neurospheres), and growing the cells in an environment that the desired cell of a different type (or "-like" cell) would be normally exposed to in vivo such as the proper temperature, pH and low oxygen environment (for example about 2-5% O_2). In various embodiments, the invention encompasses these and other related methods and techniques for facilitating cell reprogramming.

Accordingly, the method of transforming a cell of a first type into a cell of a second different type may comprise additional facultative steps. In one embodiment, the method of transforming a cell further comprises the step of pretreating the cell of a first type with a cytoskeleton disrupter. As used herein "cytoskeleton" refers to the filamentous network of F-actin, Myosin light and heavy chain, microtubules, and intermediate filaments (IFs) composed of one of three chemically distinct subunits, actin, tubulin, or one of several classes of IF protein. Accordingly, the term "cytosketeton disrupter" refers to any molecules that can inhibit the cell cytoskeleton to destabilize the cell and consequently remove the feedback mechanisms between the cell's shape and cellular and nuclear function. Suitable cytoskeleton disrupter according to the invention include, but are not limited to, the cytochalasin family of actin cytoskeleton inhibitors, such as Cytochalasin B or D, and myosin inhibitors such as 2,3-butanedione monoxime. Such pretreatment may boost reprogramming. In a preferred embodiment, the cell is cultured in the presence of at least one cytoskeleton inhibitor one day before, during, or after introducing a neurogenic transcription factor(s).

Placing the cell in conditions in conditions for supporting the transformation of the desired cell, and/or maintaining the cell in culture conditions supporting the transformation of the desired cell may comprises culturing the cell in a media comprising one or more factors appropriate for inducing the expression of the morphological and functional characteristics of the desired cell of a different type. In some embodiments the one or more factors are reprogramming factors helpful in reprogramming a cell and these reprogramming factors can be used alone or in combinations.

In other embodiments, the step of culturing the cell in a media comprising one or more factors appropriate for inducing the expression of the morphological and functional characteristics of the desired cell of a different type is carried out subsequently or simultaneously to steps iii) or iv), or subsequently or simultaneously to steps 1) or 2), as defined hereinbefore.

Those skilled in the art know many different types of media and many reprogramming factors that may be helpful in reprogramming a cell and these reprogramming factors can be used alone or in combinations. In various embodiments, the reprogramming factor is selected according to TABLE B.

In some embodiments, reprogramming factors have one or more of the following functions: decrease the expression of one or more markers of the first type of cell and/or increase the expression of one or more markers of the desired cell. Cells that exhibit a selectable marker for the desired cell are then selected and assessed for unipotency, multipotency, pluripotency, or similar characteristics (as appropriate).

In particular embodiments, the cells are cultured in serum-free medium before, during or after any one of steps i) to iv) as defined hereinbefore, or during or after steps 1) or 2), as defined hereinbefore.

In some embodiments, Mesendoderm-like cells can be created from cells such as ADSCs by up-regulating the expression of FoxD3, Mixl1, Ngn3 and MBD2. Additionally up-regulating the expression of Oct4, Sox17, Brachyury, and/or FoxA2 can be used to create Mesendoderm-like cells. In addition, media compositions detailed in Table 37 can be used to create mesendoderm-like cells from adipocyte-derived stem cells (ADSCs) and similar cells, along with the use of proper cell substrates for mesendoderm cells known in the art (e.g., gelatin-coated plates).

In some embodiments, cells such as ADSCs can be reprogrammed into pancreatic progenitor-like cells and β islet-like cells by up-regulating the expression of Sox17, Pdx1, Ngn3, and Oct4 (e.g., Oct4+Sox17+Pdx1+Ngn3; Sox17+Pdx1+Ngn3; Oct4+Pdx1+Ngn3). Additionally up-regulating the expression of FoxA2 and MBD2 can be used to create these cells. In addition, media compositions detailed in Table 39 can be used to create pancreatic progenitor-like cells and β islet-like cells from adipocyte-derived stem cells (ADSCs) and similar cells, along with the use of proper cell substrates for growing pancreatic progenitor cells and β islet-like cells known in the art (e.g., fibronectin-coated collagen gels).

In some embodiments, cells such as ADSCs can be reprogrammed into cardiac progenitor-like cells and mesoderm-like cells by up-regulating the expression of a combination of Mesp1, FoxD3, Tbx5, Brachyury (T), Nkx2.5, Sox17 and/or Gata4 (e.g., Foxd3+Sox17+Mesp1+Tbx5; Foxd3+Sox17+Mesp1+Nkx2.5; Foxd3+T+Mesp1+Gata4), which increase the expression of mesoderm and cardiac progenitors markers. Additionally up-regulating the expression of Gata6 and Baf60c can be used to create these cells. In addition, media compositions detailed in Table 44 can be used to create cardiac progenitor-like cells from adipocyte-derived stem cells (ADSCs) and similar cells, along with the use of proper cell substrates for growing cardiac progenitor cells known in the art (e.g., matrigel).

In some embodiments, cells such as ADSCs can be reprogrammed into pluripotent-like stem cells up-regulating the expression of Rex1, Oct4 and Klf4, or Sal14, Oct4, Klf4 and Nanog. Reprogramming by transient transfection of a combination of the above genes can be achieved efficiently. These cells can be differentiated into ectoderm-like cells, endoderm-like cells, or mesendoderm-like cells by methods known in the art for differentiating pluripotent stem cells into these lineages. In addition, these pluripotent-like stem cells have protective and/or therapeutic/regenerative effect on other cells (e.g., hepatocytes).

The NSLCs that is a subject of this invention have benefits over native human neural stem/progenitor cells as well as embryonic stem cells. The NSLCs do not readily form tumors or teratomas (as tested in NOD-SCID mice), can be created from easily obtainable somatic cells (e.g., fibroblasts, CD34+ blood cells, keratinocytes) and differentiated towards any specific neuronal lineage, express neurotrophic growth factors (e.g., BDNF and GDNF), express some neuronal differentiation genes while maintaining a stem cell like state allowing a higher proportion of neuronal differentiation when placed in differentiation conditions (compared to native human neural stem/progenitor cells), form functional gap junctions and readily form synapses, and are capable of attaching and surviving on 3D scaffolds and environments.

The stem-like cells of the present invention have numerous advantages over the prior art such as efficient reprogramming without gene integration or constant artificial forced gene expression, greater potency and safety over native cells (esp. stem/progenitor cells), and having the capability to be the cell of interest for a particular application (e.g., a neural stem-like cell for CNS applications; a cardiac progenitor-like cell for cardiac applications, a pancreatic progenitor-like cells or B islet-like cells for diabetes, an ectoderm-like cell, a mesoderm-like cells, an endoderm-like cell, etc.). In addition derived cells of the invention can be a relatively homogeneous population of autologous cells of a particular phenotype of interest.

The methods of the present invention allow the ability to create the native types of stem cells for particular applications (e.g., neural stem-like cells for CNS applications; cardiac progenitor-like cells for cardiac applications, pancreatic progenitor-like cells or β-like cells for diabetes, etc.) as well as creating autologous (from the patient's own cells) versions of these stem cells that allow them to graft more appropriately when delivered to the patient as a treatment or for augmenting the health or functionality of a particular tissue or organ, or for diagnostic purposes. The cells can also be used for modeling (e.g., disease modeling, or testing the effects of particular compounds or other molecules (e.g., for personalized medicine purposes)). The cells can also easily be enhanced by specific genes of interest or a defective gene repaired/replaced before delivery to the patient for treatment of a genetic disorder or for enhanced therapeutic value or augmenting the health or functionality of a particular cell, tissue, organ, system, or organism.

Obtaining Neural Stem-Like Cells (NSLCs)

According to preferred embodiments for creating Neural Stem-Like Cells (NSLCs), the methods of the invention are carried out such that cells are treated with selected agents, compounds and factors to promote the reprogramming and/or dedifferentiation towards Stem-Like Cells (SLCs). Such reprogrammed somatic cells can then be further treated with agents and/or cultured under conditions suitable for promoting reprogramming towards Neural Stem-Like Cells (NSLCs), and expansion of the NSLCs for the long-term. NSLCs according to the invention have the potential to differentiate to neuronal-like and/or glial-like cells, as well as neuronal and/or glial cells, for potential treatment of neurological diseases and injuries such as Parkinson's disease and spinal cord injury. The methods described herein are also useful for producing histocompatible cells for cell therapy.

Accordingly, some aspects of the present invention relates to generating neurons from an individual patient, thus making autologous transplantations possible as a treatment modality for many neurological conditions including neurotrauma, stroke, neurodegenerative diseases such as Multiple Sclerosis, Parkinson's disease, Huntington disease, Alzheimer's diseases. Thus, the invention provides for neurological therapies to treat the disease or trauma of interest.

Therefore, another aspect of the invention concerns a method of obtaining a Neural Stem-Like Cell (NSLC), comprising either:

1) contacting the cell of a first type with one or more neural stem cell regulating polypeptide capable of increasing intracellular levels of neural stem cell specific polypeptides within said cell and directly or indirectly remodeling the chromatin and/or DNA of the cell and driving directly or indirectly transformation of the cell of the first type into a NSLC; or
2) contacting the chromatin and/or DNA of a cell of a first type with a histone acetylator, an inhibitor of histone deacetylation, a DNA demethylator, and/or an inhibitor of DNA methylation; and increasing intracellular levels of at least one neural stem cell specific polypeptide driving directly or indirectly transformation of the cell of the first type into a NSLC.

In preferred embodiments, the step 1) comprises increasing intracellular levels of a MUSASHI1 polypeptide. As it will be explained hereinafter this can be achieved by different means including, but not limited to, transient expression of the MUSASHI1 polypeptide, preferably by transfecting an expression vector encoding the polypeptide.

In preferred embodiments, the step 2) comprises increasing intracellular levels of a MBD2 polypeptide or treating the cells with VPA and 5-AZA. As it will be explained hereinafter this can be achieved by different means including, but not limited to, transient expression of the MBD2 polypeptide, preferably by transfecting an expression vector encoding the polypeptide(s), and/or pre-treating and/or treating the cells with VPA and 5-AZA.

In one particular embodiment, reprogramming a cell of a first type to another type of cell that exhibits at least two selectable markers for neural stem cells requires transfecting the cell of a first type with one vector containing a cDNA encoding for a neurogenic transcription factor and one DNA demethylator. To enhance the de-differentiation the cells are exposed or pre-exposed to an agent(s) that inhibits DNA methylation, inhibits histone deacetylation, and/or disrupts the cell cytoskeleton. For example, the dedifferentiation can be enhanced by pre-treating the cells with an agent that disrupts the cell cytoskeleton followed by transfecting the cells with one or more vector(s) containing two neurogenic transcription factors in the presence of a DNA demethylator and/or inhibitor of DNA methylation and histone deacetylation. The histone deacetylator, inhibitor of histone deacetylation, DNA demethylator, and/or an inhibitor of DNA methylation are as defined previously.

As defined previously, the method may further comprise a preliminary step of pre-treating the cell of a first type with a cytoskeleton disrupter, as defined previously, and/or culturing the cell in a media comprising one or more reprogramming factors appropriate for appearance and maintenance of the morphological and functional characteristics of NSLCs as defined previously (e.g. a retinoid compound, a neurotrophic factor, bFGF, EGF, SHH, Wnt 3a, neuropeptide Y, Estrogen). In some embodiment the method further comprises inhibiting cellular BMP signaling pathways (e.g. by NOGGIN, fetuin, or follistatin).

In preferred embodiments, generation of a NSLC from a first cell comprises the use of one or more reprogramming agents. Suitable agents include, but are not limited to, Musashi-1 (Msi1) and Neurogenin 2 (Ngn2). Other potential agents are listed in Table A and B.

The present invention is also directed to the use of DNA expression vectors encoding a protein or transcript which upregulates the expression of neurogenesis. The genetically-engineered DNA sequence, encoding a defined reprogramming agent such as Msi1 and Ngn2, can be introduced into cells by using a mono-, bi-, or poly-cistronic vectors. The expression of an endogenous multipotency gene indicates that the cDNA encodes a protein whose expression in the cell result directly or indirectly in the de-differentiation of the cell. The newly de-differentiated mammalian cells are capable of re-differentiating to neuronal lineages to regenerate said mammalian cells, tissues, and organs.

The present invention is further directed to a method for generating NSLCs by introducing a genetically-engineered DNA sequence into human somatic cells via transient transfection. Since the DNA introduced in the transfection process is not inserted into the nuclear genome, the foreign DNA decreases over time and when the cells undergo mitosis. Nonviral vectors remain in a non-replicative form, have low immunogenicity, and are easy and safe to prepare and to use. Furthermore, plasmids may accommodate large fragments of DNA.

In one particular embodiment, the method starts with obtaining cells from the individual, and reprogramming the cells in vitro to generate NSLCs. The significant aspect of the present invention is the stable reprogramming of a somatic cell or non-neuronal cell into a NSLC that can give rise to different types of, neuronal or glial cells (including neuronal-like or glial-like cells). These can then be implanted back into the same patient from which the cells were obtained, thus making an autologous treatment modality for many neurological conditions including neurotrauma, stroke, and neurodegenerative disease possible. These can also be implanted into a different individual from which the cells were obtained. Accordingly, the cells and methods of the present invention may be helpful to treat, prevent, or to stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, or spinal cord injury. This technology provides an ample source of neural stem cells, neuroprogenitor cells, neurons and glia for clinical treatment, which can be performed by implantation of NSLCs in vivo or inducing the differentiation in vitro and implantation of neuroprogenitor cells or specific neurons or glia in vivo.

In another embodiment, the method comprises isolating somatic or non-neuronal cells and exposing the cells to one or more agents that alter cell morphology and chromatin structure, and transfecting the cells with one or more genes containing at least one cDNA encoding for a neurogenic transcription factor. The gene transfection step may be replaced with alternative agents that induce the expression of the neurogenic transcription factor(s) in the cell. Inducing epigenetic modifications to DNA and histones (especially DNA demethylation and an open chromatic structure) facilitate true reprogramming of the cells. In another embodiment, the cells are incubated in a low oxygen environment, for example 5% O_2, thereby helping in reprogramming the cells.

This methodology allows the reprogramming of a cell into a NSLC. The further course of development and the expansion of the reprogrammed cell depend on the in situ environment cues to which it is exposed. The embodiments of the invention further include growing the reprogrammed cell in an appropriate proliferation medium to expand the generated NSLC, for example Neural Progenitor proliferation Medium (StemCell Technologies) with the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), to promote the neural stem cell to proliferate.

The NSLCs obtained according to the invention can be differentiated into neuronal, astrocyte, and/or oligodendrocyte lineages in appropriate differentiation medium, for example NS-A differentiation medium (StemCell, Technologies) or NbActive medium (BrainBits™) including a retinoid compound, such as all-trans-retinoic acid or vitamin A, and BDNF, to induce the differentiation of NSLCs towards neuronal and/or glial cells. Neuronal cells include cells that display one or more neural-specific morphological, physiological, functional and/or immunological features associated with a neuronal cell type. Useful criteria features includes: morphological features (e.g., long processes or neurites), physiological and/or immunological features such as expression of a set of neuronal-specific markers or antigens, synthesis of neurotransmitter(s) such as dopamine or gamma aminobutyric acid (GABA), and functional features such as ion channels or action potentials characteristic of neurons.

In accordance with the method, reprogrammed cells can be selected based on differential adherence properties as compared to untransfected cells; for example, reprogrammed cells can form floating neurospheres or grow well on laminin while untransfected fibroblasts attach and grow well on regular cell culture treated plates. Reprogrammed cells include cells that exhibit one or more neural stem specific markers and morphology and the loss of some or all of the specific markers related to the original cells. Furthermore, some of the functionality of the neural-like cells (NLCs) can be assessed at different time points by, for example, patch-clamping, immunostaining for synaptophysin and MAP2b, and by immunochemical means such as by enzyme-linked immunosorbent assay (ELISA).

In certain embodiments, the present invention provides NSLCs that are able to initiate and direct central nervous system regeneration at a site of tissue damage and can be customized for individual patients using their own cells as the donor or starting cell. The present invention can be used to generate cells from an individual patient, thus making autologous transplantations possible as a treatment modality for many neurological conditions. Thus, this technology eliminates the problems associated with transplantations of non-host cells, such as, immunological rejection and the risk of transmitted disease. The great advantage of the present invention is that it provides an essentially limitless supply for autologous grafts suitable for transplantation. Therefore, it will obviate some significant problems associated with current source of materials and methods of transplantation.

Delivery of Polynucleotides

In certain embodiments, the invention concerns the use of polynucleotides, e.g. a polynucleotide encoding a MBD2 polypeptide, a MUSASHI1 polypeptide and/or a Ngn2 polypeptide. Means for introducing polynucleotides into a cell are well known in the art. Transfection methods of a cell such as nucleofection and/or lipofection, or other types of transfection methods may be used. For instance a polynucleotide encoding a desired polypeptide can be cloned into intermediate vectors for transfection in eukaryotic cells for replication and/or expression. Intermediate vectors for storage or manipulation of the nucleic acid or production of protein can be prokaryotic vectors, (e.g., plasmids), shuttle vectors, insect vectors, or viral vectors for example. A desired polypeptide can also be encoded by a fusion nucleic acid.

To obtain expression of a cloned nucleic acid, it is typically subcloned into an expression vector that contains a promoter to direct transcription. Suitable bacterial and eukaryotic promoters are well known in the art and described, e.g., in Sambrook and Russell (Molecular Cloning: a laboratory manual, Cold Spring Harbor Laboratory Press). The promoter used to direct expression of a nucleic acid of choice depends on the particular application. For example, a strong constitutive promoter is typically used for expression and purification. In contrast, when a dedifferentiation protein or compound is to be used in vivo, either a constitutive or an inducible promoter or compound is used, depending on the particular use of the protein. In addition, a weak promoter can be used, such as HSV TK or a promoter having similar activity. The promoter typically can also include elements that are responsive to transactivation, e.g., hypoxia response elements, Gal4 response elements, lac repressor response element, and small molecule control systems such as tet-regulated systems and the RU-486 system.

In addition to a promoter, an expression vector typically contains a transcription unit or expression cassette that contains additional elements required for the expression of the nucleic acid in host cells, either prokaryotic or eukaryotic. A typical expression cassette thus contains a promoter operably linked, e.g., to the nucleic acid sequence, and signals required, e.g., for efficient polyadenylation of the transcript, transcriptional termination, ribosome binding, and/or translation termination. Additional elements of the cassette may include, e.g., enhancers, and heterologous spliced intronic signals.

Expression vectors containing regulatory elements from eukaryotic viruses are often used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 late promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Standard transfection methods can be used to produce bacterial, mammalian, yeast, insect, or other cell lines that express large quantities of dedifferentiation proteins, which can be purified, if desired, using standard techniques. Transformation of eukaryotic and prokaryotic cells is performed according to standard techniques.

Any procedure for introducing foreign nucleotide sequences into host cells can be used. These include, but are not limited to, the use of calcium phosphate transfection, DEAE-dextran-mediated transfection, polybrene, protoplast fusion, electroporation, lipid-mediated delivery (e.g., liposomes), microinjection, particle bombardment, introduction of naked DNA, plasmid vectors, viral vectors (both episomal and integrative) and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (see, e.g., Sambrook et al., supra). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the protein of choice.

Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids into mammalian cells or target tissues. Such methods can be used to administer nucleic acids encoding reprogramming polypeptides to cells in vitro. Preferably, nucleic acids are administered for in vivo or ex vivo gene therapy uses. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell.

Methods of non-viral delivery of nucleic acids include lipofection, microinjection, ballistics, virosomes, liposomes, immunoliposomes, polycation or lipid-nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids suitable for efficient receptor-recognition lipofection of polynucleotides are known. Nucleic acid can be delivered to cells (ex vivo administration) or to target tissues (in vivo administration). The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to those of skill in the art.

The use of RNA or DNA virus-based systems for the delivery of nucleic acids take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (in vivo) or they can be used to treat cells in vitro, wherein the modified cells are administered to patients (ex vivo). Conventional viral based systems for the delivery include retroviral, lentiviral, poxviral, adenoviral, adeno-associated viral, vesicular stomatitis viral and herpesviral vectors, although integration in the host genome is possible with certain viral vectors, including the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

pLASN and MFG-S are examples of retroviral vectors that have been used in clinical trials. In applications for which transient expression is preferred, adenoviral-based systems are useful. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and are capable of infecting, and hence delivering nucleic acid to, both dividing and non-dividing cells. With such vectors, high titers and levels of expression have been obtained. Adenovirus vectors can be produced in large quantities in a relatively simple system.

Gene therapy vectors can be delivered in vivo by administration to an individual patient, typically by systemic administration (e.g., intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application. Alternatively, vectors can be delivered to cells ex vivo, such as cells explanted from an individual patient (e.g., lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have been reprogrammed.

Ex vivo cell transfection for diagnostics, research, or for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject organism, transfected with a nucleic acid (gene or cDNA), and re-infused back into the subject organism (e.g., patient). Various cell types suitable for ex vivo transfection are well known to those of skill in the art.

Vectors (e.g., retroviruses, adenoviruses, liposomes, etc.) containing therapeutic nucleic acids can be also administered directly to the organism for transfection of cells in vivo. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention.

Delivery of Polypeptides

In most, if not all the methods described herein, an alternative possibility consists of bypassing the use of a polynucleotide and contacting a cell of a first type cell directly with a compound (e.g. a polypeptide) for which an increased intracellular level is desired. In other embodiments, for example in certain in vitro situations, the cells are cultured in a medium containing one or more functional polypeptides.

An important factor in the administration of polypeptides is ensuring that the polypeptide has the ability to traverse the plasma membrane of a cell, or the membrane of an intracellular compartment such as the nucleus. Cellular membranes are composed of lipid-protein bilayers that are freely permeable to small, nonionic lipophilic compounds and are inherently impermeable to polar compounds, macromolecules, and therapeutic or diagnostic agents. However, proteins, lipids and other compounds, which have the ability to translocate polypeptides across a cell membrane, have been described. For example, "membrane translocation polypeptides" have amphiphilic or hydrophobic amino acid subsequences that have the ability to act as membrane-translocating carriers. Polypeptides for which an increased intracellular level is desired according to the invention can be linked to suitable peptide sequences for facilitating their uptake into cells. Other suitable chemical moieties that provide enhanced cellular uptake can also be linked, either covalently or non-covalently, to the polypeptides. Other suitable carriers having the ability to transport polypeptides across cell membranes may also be used.

A desired polypeptide can also be introduced into an animal cell, preferably a mammalian cell, via liposomes and liposome derivatives such as immunoliposomes. The term "liposome" refers to vesicles comprised of one or more concentrically ordered lipid bilayers, which encapsulate an aqueous phase. The aqueous phase typically contains the compound to be delivered to the cell. In certain embodiments, it may be desirable to target a liposome using targeting moieties that are specific to a particular cell type, tissue, and the like. Targeting of liposomes using a variety of targeting moieties (e.g., ligands, receptors, and monoclonal antibodies) has been previously described.

Cells and Cell Lines

The invention encompasses the cells, cell lines, stem cells and purified cell preparations derived from any of the methods described herein. In some embodiments, the cells, cells lines, stem cells and purified cells preparations of the invention are of mammalian origins, including but not limited to human, primates, rodent, dog, cat, horse, cow, or sheep. In preferred embodiments, they originate from a human.

Accordingly, another aspect of the invention relates to modified cells, cell lines, pluripotent, multipotent or unipotent cells and purified cell preparations, wherein any of these cells comprise an exogenous polynucleotide encoding Musashi1 (Msi1); Msi1 and Ngn2; Msi1 and MBD2; and Ngn2 and MBD2; Msi1, Ngn2 and MBD2; Msi1, Ngn2, Nestin and MBD2; and other potential combinations from Table A preferably including Msi1 and Ngn2 and MBD2. In preferred embodiments the cell according to the invention is a stem-like cell, more preferably a Neural Stem-Like Cell (NSLC), the cell possessing one or more of the following characteristics:

- expression of one or more neural stem cell marker selected from the group consisting of Sox2, Nestin, GFAP, Msi1, and Ngn2;
- decreased expression of one or more genes specific to the cell that the NSLC was obtained from (e.g. see Table C);
- forms neurospheres in the neurosphere colony formation assay;
- capable of being cultured in suspension or as an adherent culture;
- capable of proliferating without the presence of an exogenous reprogramming agent for over 1 month, preferably over 2 months, over 3 months, over 5 months and even for more than a year;
- capable of dividing every 36 hours at low passage;
- positive for telomerase activity;
- capable of differentiation into a neuronal-like cell, astrocyte-like cell, oligodendrocyte-like cell and combinations thereof;
- decreased expression of telomerase and one or more neural stem cell markers after differentiation;
- having one or more morphological neurite-like processes (axons and/or dendrites) greater than one cell diameter in length after differentiation into a neuronal-like cell;
- expression of at least one neural-specific antigen selected from the group consisting of neural-specific tubulin, microtubule associated protein 2, NCAM, and marker for a neurotransmitter after differentiation into a neuronal-like cell;
- expression of one or more functional neural markers (e.g. synapsin) after differentiation into a neuronal-like cell;
- capable of releasing one or more neurotrophic factors (e.g. BDNF) after differentiation into a neuronal-like cell;
- negative in a tumor colony forming assay;
- negative for tumor growth in SCID mice;
- negative for teratoma growth in SCID mice;
- capable of significantly improving one or more functional measures after placement of an adequate number of NSLCs into the void in a brain ablation model;
- capable of significantly improving or maintaining one or more functional measures after injecting an adequate number of NSLCs into an EAE model; and
- capable of improving one or more functional measures more significantly than hNPCs in CNS injury or neurodegenerative models.

Examples of all of the above items can be found in the Examples section of this application.

In preferred embodiments, a NSLC according to the inventions possesses all of the following characteristics:

- ability to self-renew for significantly longer than a somatic cell;
- is not a cancerous cell;
- is stable and not artificially maintained by forced gene expression or by similar means and may be maintained in standard neural stem cell media;

can differentiate to a progenitor, precursor, somatic cell or to another more differentiated cell type of the same lineage;

has the characteristics of a stem cell and not just certain markers or gene expression or morphological appearance; and

does not exhibit uncontrolled growth, teratoma formation, and tumor formation in vivo.

In one particular embodiment, the reprogrammed cells (NSLCs) according to the invention are capable of proliferating for several months without losing their neural stem cell markers and their ability to differentiate towards neuron-like, astrocyte-like, and oligodendrocyte-like cells. The generation of the neural lineages is characterized based on morphology, phenotypic changes and functionality.

In some embodiments, the cells of the invention may have one or more of the following characteristics and properties: self-renewal, multilineage differentiation in vitro and in vivo, clonogenicity, a normal karyotype, extensive proliferation in vitro under well defined culture conditions, and the ability to be frozen and thawed, as well as any of the commonly known and/or desired properties or characteristics typical of stem cells. The cells of the invention may further express molecular markers of multipotent or pluripotent cells (i.e. gene and surface markers as defined previously).

Another aspect of the invention relates to the production of tissue specific autologous (self) stem and/or progenitor cells. These stem and/or progenitor cells may be used in cell therapy applications to treat diseases of cellular degeneration. Diseases of cellular degeneration include, for example, neurodegenerative diseases such as stroke, Alzheimer's disease, Parkinson's disease, multiple sclerosis, Amyotrophic lateral sclerosis, macular degeneration, osteolytic diseases such as osteoporosis, osteoarthritis, bone fractures, bone breaks, diabetes, liver injury, degenerative diseases, myocardial infarct, burns and cancer. It is envisioned that cells according to the invention may be implanted or transplanted into a host. An advantage of the invention is that large numbers of autologous stem cells can be produced for implantation without the risk of immune system mediated rejection. Those cells can lead to production of tissue suitable for transplant into the individual. Since the tissue is derived from the transplant recipient, it should not stimulate an immune response, as would tissue from an unrelated donor. Such transplants can constitute tissues (e.g. vein, artery, skin, muscle), solid organ transplants (e.g., heart, liver, kidney), neuronal cell transplants, or bone marrow transplants such as are used in the treatment of various malignancies such as, for example, leukemias and lymphomas. Neural stem cell, neuroprogenitor, or neuronal cell (as well as NSLCs and derivations thereof) transplants can also be used in the treatment of, for example, neurological disorders, stroke, spinal cord injury, Parkinson's disease, and the like, as well as potentially some non-neurological disorders such as a cardiac infarct.

Another aspect of the invention relates to a method to produce ex vivo engineered tissues for subsequent implantation or transplantation into a host, wherein the cellular components of those engineered tissues comprise cells according to the invention, or cells derived therefrom. For example, expanded cultures of the cells of the invention may be differentiated by in vitro treatment with growth factors and/or morphogens. Populations of differentiated cells are then implanted into the recipient host near the site of injury or damage, or cultured in vitro to generate engineered tissues, as described.

The methods and cells of the invention described herein can be used to immortalize cells, for example to generate a cell line. Using the methods disclosed herein, a somatic cell can be transformed into one possessing a dedifferentiated phenotype, thereby facilitating the generation of cell lines from a variety of tissues. Therefore, the invention encompasses such immortalized cells.

In addition, the methods of deriving the cells according to the invention, may be helpful in scientific and therapeutic applications including, but not limited to, (a) scientific discovery and research involving cellular development and genetic research (e.g. uses in lieu of human stem cells as a model cell line to study the differentiation, dedifferentiation, or reprogramming of human cells), (b) drug development and discovery (e.g., screening for efficacy and toxicity of certain drug candidates and chemicals, screening for prospective drugs or agents which mediate the differentiation, dedifferentiation, or reprogramming of cells), (c) gene therapy (e.g., as a delivery device for gene therapy), and (d) treatment of injuries, trauma, diseases and disorders including, but not limited to, Parkinson's, Alzheimer's, Huntington's, Tay-Sachs, Gauchers, spinal cord injury, stoke, burns and other skin damage, heart disease, diabetes, Lupus, osteoarthritis, liver diseases, hormone disorders, kidney disease, leukemia, lymphoma, multiple sclerosis, rheumatoid arthritis, Duchenne's Muscular Dystrophy, Ontogenesis Imperfecta, birth defects, infertility, pregnancy loss, and other cancers, degenerative and other diseases and disorders.

Additional aspects concern therapeutic methods, methods of treatment and methods of regenerating a tissue or organ in a mammal (e.g. a human subject). One particular method concerns a method of regenerating a mammalian tissue or organ which comprises contacting the tissue or organ to be regenerated with a SLC, NSLC, or other desired cell or artificial tissue construct as defined herein. The SLC, NSLC, desired cell or artificial tissue construct may be placed in proximity to the tissue or organ to be regenerated by administering to the subject using any suitable route (e.g. injecting the cell intrathecally, directly into the tissue or organ, or into the blood stream).

Another method for repairing or regenerating a tissue or organ in a subject in need thereof comprises administering to the subject a compound inducing a direct or indirect endogenous expression of at least one gene regulator in cells of the tissue or organ and/or a compound inducing a direct or indirect endogenous expression of at least one gene regulator in cells capable of transformation or dedifferentiation in vivo in the subject. Accordingly, the expression of the at least one gene regulator reprograms the cells into desired cells of a different type (e.g. neural stem-like cells), and these cells of a different type are effective in repairing or regenerating said tissue or organ.

Another method comprises obtaining cells or tissue from a patient (e.g. hematopoietic stem cells, fibroblasts, or keratinocytes), reprogramming a plurality of such cells or the tissue, and reintroducing the reprogrammed cells or tissue into the patient. A related aspect concerns pharmaceutical compositions comprising a plurality of a desired cell, SLC and/or Neural Stem-Like Cell (NSLC) or reprogrammed tissue as defined herein.

The therapeutic methods of the invention may be applicable to the regeneration or repair of various tissues and organs including, but not limited to, the brain, the spine cord, the heart, the eye, the retina, the cochlea, the skin, muscles, intestines, pancreas (including beta cells), kidney, liver, lungs, bone, bone marrow, cartilage, cartilage discs, hair follicles, teeth, blood vessels, glands (including endocrine and exocrine glands), ovaries, reproductive organs, mammary and breast tissue.

A related aspect concerns pharmaceutical compositions comprising a plurality of desired cell, SLC and/or Neural Stem-Like Cell (NSLC) as defined herein.

Tissues

Another aspect of the invention relates to a tissue containing reprogrammed cells as defined herein that can be implanted into a subject in need thereof.

In some embodiments the present invention provides for the reprogramming of cells within a tissue, for example an in vitro produced 3D tissue construct comprising cells and extracellular matrix produced by these cells. In addition, transfected cells can be seeded on top of these 3D tissue constructs that can be made completely autologously, thus preventing host rejection, making it completely immunocompatible and as carrier for reprogrammed cells to be transplanted in vivo. Advantageously, these newly created cells can be used in their undifferentiated and/or differentiated state within these tissues for in vitro diagnostic purposes or transplanted into a patient in need of such a construct in cell therapy/tissue replacement approaches.

The invention further encompasses 3D Neuronal-Like multilayer tissue. Cells within CDM reprogrammed to Neural Stem-Like Cells according to the invention readily differentiate into neuronal-like cells, astrocyte-like cells, and oligodendrocyte-like cells within the CDM. It is thus possible to use CDM and reprogramming methods of the invention to reprogram the cells within the CDM to form 3D Neuronal-Like multilayer tissue (up to >30 cell layers). Such 3D tissue comprises neurons (or specifically, neuron-like cells), astrocytes (or specifically, astrocyte-like cells), and oligodendrocytes (or specifically, oligodendrocyte-like cells) and it can be made completely autologously, can be manually handled and implanted with relative ease, or can used as an in vitro CNS tissue model.

One particular aspect concerns an artificial tissue construct which comprises a 3D assembly of in vitro cultured cells and extracellular matrix produced by these cells. The cells may be desired cells, SLC and/or a plurality of Neural Stem-Like Cell (NSLC) obtained using any one of the methods described herein.

Screening Methods

Another aspect of the invention relates to methods for identifying new compounds (e.g. small molecules, drugs, etc) capable of transforming a cell of a first type to a desired cell of a different type. These new compounds may be useful for research purposes or as medicaments for use in repairing or regenerating tissues in a subject.

The Examples section provides principles, methods and techniques useful for screening and identifying such desirable active compounds. For instance, those skilled in the art will understand that it is conceivable to screen for compounds that will induce transformation of a cell of a first type to a NSLC by replacing the “induction” or “biological activity” provided by the transient increase of Musashi1, NGN2 or MBD2 in the cell by a candidate compound to be tested (e.g. a library of small molecules or compounds) and measuring activity or efficacy of the candidate compound in generating the NSLC. Individual or mixture of active compounds would be selected if they have the same activity and/or if they can provide the same or similar effects as these polypeptides (e.g. cell transformation and/or appearance of any desirable markers or desirable characteristics as defined hereinbefore). For example, a compound or mixture of compounds capable of transforming a fibroblast into a NSLC could be identified by:

(i) Setting up, culturing and transforming the fibroblasts into NSLC as in Example 1;

(ii) Screening a library of compounds by replacing Msi1, Ngn2 and/or MBD2 with each candidate compound in a different well;

(iii) Identify a compound ‘hit’ when the candidate compound is able to transform the fibroblasts into NSLCs approximately as well as the replaced Msi1, Ngn2 and/or MBD2;

(iv) If compound from part (iii) did not replace all of Msi1, Ngn2 and MBD2, and is not able to transform the fibroblasts into NSLCs by itself, then by including the compound from (iii) in each well, screening a library of compounds by replacing the Msi1, Ngn2 and/or MBD2 that was not removed in part (ii) with each candidate compound in a different well;

(v) Identify a compound ‘hit’ when the candidate compound is able to transform, along with the compound from part (iii), the fibroblasts into NSLCs approximately as well as the replaced Msi1, Ngn2 and/or MBD2;

(vi) If compound from part (V) did not replace all of Msi1, Ngn2 and/or MBD2, and is not able to transform the fibroblasts into NSLCs together with the compound from part (iii), then by including the compound from (iii) and (v) in each well, screening a library of compounds by replacing the Msi1, Ngn2 or MBD2 that was not removed in part (ii) and (iv) with each candidate compound in a different well;

(vii) Identify a compound ‘hit’ when the candidate compound is able to transform, along with the compound from part (iii) and (v), the fibroblasts into NSLCs approximately as well as the replaced Msi1, Ngn2 or MBD2;

(viii) A combination of the compounds from part (iii), (v) and (vii) will be able to transform the fibroblasts into NSLC; modifications to these compounds can be made and further screened to identify more effective or safe versions of these compounds.

The same principles are applicable for other desired types of stem-like cells including pluripotent-like cells, mesendoderm-like cells, pancreatic progenitor-like cells, etc. Tables A and B, and the Examples section provides, for each of these types of cells, a list of potential genes and/or compounds to be considered in such screening methods.

Accordingly, the present invention encompasses these and any equivalent screening methods where candidate compounds are tested for their efficacy in transforming a cell of a first type to a desired cell of a different type when compared to the efficacy of the reprogramming factor and/or gene regulator as defined herein.

Delivery of Neurotrophic Factors

Local delivery of neurotrophic factors has been suggested as a method to treat several neurological conditions. Strategies using neurotrophic molecules focus on preventing the progressive loss of neurons, maintaining neuronal connections and function (neuroprotection), and inducing additional regenerative responses in neurons such as increased neurotransmitter turnover and/or axonal sprouting (neuroregeneration). Up to date, several therapeutic strategies to deliver neurotrophic-factors in animal models have been explored, but so far testing of the effects of growth factors on the brain and nervous system have been limited to direct peripheral injection of large doses of these factors, which carries a significant risk of side effects. Accordingly, a related aspect of the invention relates to overcoming these problems by using NSLC cells and cell lines according to the invention which can stably express and secrete growth factors of potential interest after transplantation.

To summarize, the present invention provides a plentiful source of Neural Stem-Like Cells, Neuron-Like Cells, Astrocyte-Like Cells or Oligodendrocyte-Like Cells for potential clinical treatments which require transplantation of neural stem cells, neurons, astrocytes or oligodendrocytes 1) to compensate for a loss of host cells (ex, neurons) or 2) as vehicles to deliver genetically-based drugs. Further, the invention provides a novel neurological tool for use in basic research and drug screening.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures, embodiments, claims, and examples described herein. Such equivalents are considered to be within the scope of this invention and covered by the claims appended hereto. The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

The examples set forth herein below provide exemplary methods for obtaining Reprogrammed and Dedifferentiated cells, including Neural Stem-Like Cells (NSLCs). Also provided are exemplary protocols, molecular tools, probes, primers and techniques.

Example I

Preparation of Human Fibroblast Cells

Human Foreskin fibroblast (HFF) cells were purchased from American Type Culture Collection (ATCC, Manassas, Va.) and expanded in cell culture flasks with Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (FCS, Hyclone Laboratories), 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate (Invitrogen) at 37° C., 5% CO_2. The medium was changed twice per week. Cells were trypsinized using Trypsin 0.25% for 4 minutes at 37° C., followed by adding trypsin inhibitor solution, pelleting the cells by centrifugation, washing the cells once with PBS, and plating the cells at a ratio of 1:2 onto tissue culture flasks until a suitable number of cells was reached.

Cells were then trypsinized and plated (8×10^4 cells/well) in cell culture plates pre-coated with Laminin (10 μg/ml, Sigma) in two different composition of CDM medium: CDM I Medium consisting of a 3:1 ratio of Dulbecco's modified Eagle medium (DMEM, high glucose (4.5 g/L) with L-glutamine and sodium pyruvate) and Ham's F-12 medium supplemented with the following components: EGF (4.2×10^{-10}M), bFGF (2.8×10^{-1}M), ITS (8.6×10^{-5}M), dexamethasone (1.0×10^{-7}M), L-3,3',5-triiodothyronine (2.0×10^{-10}M), ethanolamine (10^{-4}M), GlutaMAX™ (4×10^{-3}M), and glutathione (3.3×10^{-6}M), but without the presence of L-ascorbic acid.

CDM II Medium consisting of a 3:1 ratio of Dulbecco's modified Eagle medium (DMEM, high glucose (4.5 g/L) with L-glutamine and sodium pyruvate) and Ham's F-12 medium supplemented with the following components: EGF (2.5 ng/ml), bFGF (10 ng/ml), ethanolamine (2.03 mg/ml), insulin (10 mg/ml), Selenious acid (2.5 μg/ml), dexamethasone (19.7 μg/ml), L-3,3',5-triiodothyronine (675 ng/ml), GlutaMAX™ (4×10^{-3}M), and glutathione (3.3×10^{-6}M).

Transient Transfection of HFF by Lipofectamine Using Constructed Vectors

After two days in culture, cells were transfected with pCMV6-XL5-MBD2 (2 μg) (a DNA demethylator) using lipofectamine reagent (Invitrogen) as per the manufacturer's protocol. The DNA-lipid complex was added to cells and incubated for 24 h at 37° C., 5% CO_2. After 24 hours of transfection with the DNA demethylator, the medium was changed and cells were transfected by pCMV6-XL5-Musashi1 (2 μg, Origene) or pCMV6-XL4-Ngn2 (2 μg, Origene) for 24 h. After 24 hours, the medium was changed to Neural Progenitor Basal Medium (NPBM, Lonza) supplemented with Noggin (20 ng/ml, Peprotech), EGF (20 ng/ml, Peprotech), and bFGF (20 ng/ml, Peprotech) and cultured in this Proliferation Medium. Cells were retransfected after three days and incubated at 37° C., 5% CO_2 and 5% O_2. After 7 days in proliferation conditions, 50% of the Proliferation Medium was changed to Differentiation Medium (NbActive, BrainBits™) supplemented with Forskolin (10 μM, Tocris), all-trans-Retinoic Acid (ATRA, 5 μM, Spectrum), bFGF (20 ng/ml, Peprotech), NGF (20 ng/ml, Peprotech), and BDNF (20 ng/ml, Peprotech); medium was changed every day by increasing the percentage of Differentiation Medium over Proliferation Medium, and the cells were cultured for 20 days.

Visual observation of reprogrammed cells was performed by light microscopic observation every day following transfection using bright field at 10× magnification. Samples were collected at different time points (6, 12, and 20 days) to analyze neuronal gene expression and protein levels by gene array and immunohistochemistry. Following transfection, reprogramming cells displayed a rapid change in cellular morphology within 3 days post-transfection. The cells were more rounded and the cell's cytoplasm retracted towards the nucleus forming contracted cell bodies with extended cytoplasmic extensions and exhibiting neuronal perikaryal appearance at day 6 and 12, which was maintained until day 20. However, this morphology was not observed in untransfected cells at day 6 and 12.

Gene Array Analysis

Characterization of the newly engineering cells after transfection was performed using a neuronal gene-array containing 48 partial cDNAs coding for these genes and controls.

RNA was isolated from samples using QIAshredder™ (Qiagen) and RNeasy™ Plus mini Kit (Qiagen) as per manufacturer's instructions. DNase I treatment was performed on the RNeasy™ Column to further remove the transfected plasmid DNA using Rnase-Free DNase Set (Qiagen). RNA was eluted in 35 μl of RNase-free water. Before cDNA synthesis, all RNA samples were quantified using the NanoDrop 1000™ (ThermoScientific). cDNA was prepared using the High Capacity cDNA archive kit (Applied Biosystems) as per the manufacturer's instructions. 400 ng of RNA was used in each 500 μl RT reaction. The resulting cDNA samples were used immediately for TLDA analysis. For each card of the Taqman™ low-density array (TLDA), there are eight separate loading ports that feed into 48 separate wells for a total of 384 wells per card. Each 2 μl well contains specific, user-defined primers and probes, capable of detecting a single gene. In this study, a customized Neuronal Markers 2 TLDA was configured into eight identical 48-gene sets, i.e. 1 loading port for each 48-gene set. Genes were chosen based on literature. Each set of 48 genes also contains three housekeeping genes: ACTIN, GAPDH, and PPIA.

A sample-specific master mix was made for each sample by mixing cDNA (160 ng for each loading port), 2×Taqman™ Gene Expression Master Mix (Applied Biosystems) and nuclease-free water (USB) for a total of 100 μl per loading port. After gentle mixing and centrifugation, the mixture was then transferred into a loading port on a TLDA card. The array was centrifuged twice for 1 minute each at 1200 rpm to distribute the samples from the loading port into each well. The card was then sealed and PCR amplification was performed using Applied Biosystems 7900HT™ Fast Real-time PCR system. Thermal cycler conditions were as follows: 2 minutes at 50° C., 10 minutes at 94.5° C., and 30 seconds at 97° C., 1 minute at 59.7° C. for 40 cycles. 1 TLDA's was prepared for 8 samples.

Relative Expression values were calculated using the Comparative C_T method. Briefly, this technique uses the formula $2^{-\Delta\Delta CT}$ to calculate the expression of target genes normalized to a calibrator. The threshold cycle (C_T) indicates the cycle number at which the amount of amplified target reaches a fixed threshold. C_T values range from 0 to 40 (the latter representing the default upper limit PCR cycle number that defines failure to detect a signal). ΔC_T values [ΔC_T=C_T (target gene)−C_T (Average of 3 Housekeeping genes)] were calculated for HFF Ctrl, and subsequently used as the calibrator for the respective samples. All gene expression values were assigned a relative value of 1.00 for the calibrator, which is used to determine comparative gene expression such that $\Delta\Delta C_T$=ΔC_T (Treated)−ΔC_T (HFF Ctrl). Relative Expression is calculated using the formula $2^{-\Delta\Delta CT}$.

Quantitative comparison of astrocyte, neuron, and oligodendrocyte gene expression allowed identification of the majority of the genes that are differentially expressed in reprogrammed cells. Data in Table 1 were analyzed by using a significance analysis algorithm to identify genes that were reproducibly found to be enriched in reprogrammed cells compared to untransfected cells. After the transfection with Msi1 or Ngn2 in the presence of MBD2, the expression of oligodendrocytes progenitors such as NKx2.2, olig2, and MAG and two markers for astrocytes (GFAP and AQP4) were highly increased. Also, several markers of early neuronal cells were enhanced after the transfection of HFF. TDLA data revealed a remarkable increase in specific markers for interneurons, such as somatostatin and calbindin1. The induction of Doublecortin (DCX), which is expressed by migrating immature cells during development, and acetylcholine (ACHE) mRNA, an early marker of neuronal cells, were highly expressed in the reprogrammed cells (Table 1). Transfection increased the expression of dihydropyrimidinase-like 3 (DPYSL3), an early marker of newborn neurons, to fivefold with Msi1 and seven fold with Ngn2. Expression of Microtubule-Associated Protein 2 (MAP2), an essential marker for development and maintenance of early neuronal morphology and neuronal cell adhesion molecule, were highly expressed with Msi1 and Ngn2 (Table 1). The expression of enolase-2, a marker of mature neurons, was 20-fold enhanced by Msi1 and Ngn2. Member of the NeuroD family NeuroD1 was highly expressed after transfection with Msi1 to 84 fold and to 34 by Ngn2.

Gene expression of growth factors such as IGF-1, IGF2, NPY and CSF-3 was also enhanced in reprogrammed cells. The expression of VEGF and GDNF genes were up-regulated to almost five fold and seven fold by Msi1 and Ngn2, respectively. However, the expression of BDNF, EGF, and bFGF were not activated and even down-regulated as compared to untransfected cells. The expression of growth associated protein (GAP-43), a growth- and regeneration-associated marker of neurons, and expression of netrin, implicated in neuronal development and guidance, were highly enriched in reprogrammed cells. Expression of receptors for growth and neurotrophin factors was increased, such as type III receptor tyrosine kinase, neurotrophic tyrosine kinase, and neurotrophic tyrosine kinase receptor. Vimentin and fibronectin, markers for fibroblasts, were down-regulated in reprogrammed cells compared to the untransfected control fibroblast cultures.

TABLE 1

Gene array of transfected human fibroblast cells by Msi1/MBD2 and Ngn2/MBD2. Gene array was performed on samples after two weeks of differentiation. Expression values are given relative to untransfected fibroblasts

Symbol	Common name and description	Company Gene ID	Relative expression to Msi1	Relative expression to Ngn2
Astrocytes and oligodendrocytes markers				
NKx2-2	Markers for oligodendrocyte progenitors	NM_002509.2	very high	very high
OLIG2	Oligodendrocyte lineage transcription factor 2	NM_005806.2	47.511	8.38
MAG	Myelin-associated glycoprotein	NM-080600.1	212.61	4.51
GFAP	Glial fibrillary acidic protein	NM_002055.4	very high	very high
AQP4	Aquaporin 4	NM_001650.4	83.77	56.86
NC markers				
SST	Somatostatin, specific marker for interneurons	NM_001048.3	32.73	35.34
CALB1	Calbindin 1, interneuron marker	NM_004929.2	18.21	13.22
Tubulin1A	Are necessary for axonal growth	NM_006009.2	7.45	9.32
NES	Precursor neurons (nestin)	NM_006617.1	1.61	1.54
DCX	An early neuronal marker (Doublecortin)	NM_178151.1	very high	very high
ACHE	Acetylcholinesterase, marker of early neuronal development	NM_015831.2	9.02	13.22
ENO2	A marker for neurons cells, enolase	NM_001975.2	22.62	20.68
NEUROD1	Neural marker; expression gradually increased from neural precursor to fully differentiated neuron	NM_002500.2	84.22	34.27
DPYSL3	Dihydropyrimidinase-like3, marker of immature neurons	NM_001387.2	5.33	7.02

TABLE 1-continued

Gene array of transfected human fibroblast cells by Msi1/MBD2 and Ngn2/MBD2. Gene array was performed on samples after two weeks of differentiation. Expression values are given relative to untransfected fibroblasts

Symbol	Common name and description	Company Gene ID	Relative expression to Msi1	Relative expression to Ngn2
MAP2	Microtubule-associated protein 2, essential for development of early neuronal morphology and maintenance of adult neuronal morphology	NM_002374.3	86.38	89.67
NCAM	Neural cell adhesion molecule 1	NM_18135.3	very high	very high
CEND1	Cell cycle exit & neuronal differentiation, early marker of proliferating precursor cells that will differentiate to neurons	NM_016564.3	4.80	5.57
Neuroregeneration and survival genes				
FGF2	Fibroblast growth factor	NM_002006.4	0.06	0.11
EGF	Epidermal growth factor,	Hs00153181_m1	0.99	0.56
IGF-1	Insulin growth factor-1,	NM_000618.2	58.92	21.21
IGF-2	Insulin growth factor-2	NM_0000612.3	very high	very high
CSF3	Granulocyte colony-stimulating factor	NM_2219.1	very high	42.60
BDNF	Brain derived growth factor, neurogenesis	NM-199231.1	0.05	0.03
GDNF	Glial derived neurotrophic factor	NM-000614.2	4.77	6.89
CNTF	Ciliary neurotrophic factor	NM_001025366.1	1.86	1.09
VEGF	Vascular endothelial growth factor		6.67	7.32
BMP-4	Bone morphogenetic protein 4	NM_130850.1	5.96	8.57
KDR	Type III receptor tyrosine kinase)	NM_002253.1	31.78	6.83
NTRK2	Neurotrophic tyrosine kinase receptor (TrkB)	NM_006180.3	10.31	13.37
NPY	Neuropeptide factors	NM_00905.2	very high	very high
PIK3CG	phosphoinositide-3-kinase,	NM_002649.2	very high	very high
STAT3	Signal transduction transcription 3	NM_213662.1	2.14	3.65
Gap43	Growth associated protein 43	NM_002045.2	very high	very high
NTN1	Netrin1, implicated in neuronal development and guidance	_NM_006180.3	26.84	23.98
NTRk2	Neurotrophic tyrosine kinase, receptor, type 2	NM_024003.1	10.31	13.37
Slit	Axonal guidance molecules	NM_003061.1	very high	very high
Vimentin	Radial glia and fibroblast marker	Hs00185584	0.11	0.13
Fibronectin	fibronectin is a marker for fibroblasts	NM_212474.1	0.15	0.23

Immunohistochemical Analysis

Cells were fixed with a 4% formaldehyde/PBS solution for 10 min at room temperature and subsequently permeabilized for 5 min with 0.1% Triton X100™ in 4% formaldehyde/PBS. After two brief washes with PBS, unspecific antibody binding was blocked by a 30 min incubation with 5% normal goat serum in PBS. Then primary antibodies were added in 5% normal goat serum/PBS as follows: Mouse anti-Nestin (1:100, BD) as an intermediate microfilament present in neural stem cells and mouse anti-NCAM (1:100, Neuromics) as neuronal adhesion molecule. After a 2 h incubation the cells were washed 4 times for 5 min each with 0.1% Tween™/PBS. Appropriate fluorescence-tagged secondary antibody was used for visualization; Goat anti-mouse 546 (1:200, invitrogen) prepared in 5% normal goat serum/PBS was used. After incubation for one hour, cells were washed in 0.1% Tween™/PBS three times for 5 min each. The DNA stain Hoechst33342 (Invitrogen) was used as a marker of nuclei (dilution 1:5000 in PBS, 10 min incubation). Fluorescence images were taken with a Cellomics™ ArrayScan HCS Reader microscopy system. To determine an estimate of the percentage of cells adopting neuronal or glial phenotypes, random fields were selected and for each field the total number of cells (as determined by counting Hoechst stained nuclei) and the total number of cells positive for neuronal or glial markers were determined.

To confirm that these cells exhibited markers of neuronal lineages, cells were immunostained for nestin and NCAM. This analysis revealed that reprogrammed cells expressed both proteins. NCAM was present in cells during the 6 days post-transfection and increase at day 12 and 20 following differentiation, while the inverse pattern was observed for the nestin staining.

This study showed the ability to reprogram HFF cells using one neurogenic transcription factor with the presence of a DNA demethylator towards cells that expressed neuronal genes and proteins specific to neural stem cells and neuronal cells. These reprogrammed cells were stable in culture for at least 2 weeks.

Example II

Comparison of Reprogramming Efficiency of Three Different Neurogenic Genes

HFF cells were cultured as described in Example I and plated in CDM I medium. Cells were transfected using the Amaxa Nucleofector™ Device (Lonza). The HFFs were harvested with TrypLE™ (Gibco), resuspended in CDM Medium and centrifuged for 10 min at 90×g (1×10^6 cells/tube). The supernatant was discarded and gently resuspended in 100 μl of Basic Nucleofector™ Solution (basic Nucleofector™ kit for primary mammalian fibroblasts, Lonza). Each 100 μl of cell suspension was combined with a different mix of plasmid DNA (for example, sample 1 was mixed with 2 μg of pCMV6-XL5-Pax6 and 2 μg pCMV6-XL5-MBD2). Cell suspension was transferred into an Amaxa certified cuvette and transfected with the appropriate program (U023). The sample was transferred without any further resuspension into a coated culture plate with LAS-Lysine/Alanine (BrainBits™, 50 μg/ml) and the cells were incubated at 37° C., 5% CO_2. These steps were repeated for each sample that was transfected. After 24 hours, the medium was changed to Proliferation Medium. After two days, cells were retransfected using lipofectamine as described in Example I and incubated at 37° C., 5% CO_2 and 5% O_2. After 6 days, differentiation was induced with Differentiation Medium that gradually replaced the Proliferation Medium over several days. Cells were collected at day 14 for RT-PCR and immunohistochemistry analysis.

Gene Expression Analysis

RNA isolation and quantification was performed as previously described in Example I. cDNA was prepared using the High Capacity cDNA RT kit (Applied Biosystems) as per the manufacturer's instructions with a final cDNA concentration of 2 ng/μl. Real-time PCR was then performed for each gene of interest using the FAST PCR master mix (Applied Biosystems) and the Taqman™ Gene Expression Assays (Applied Biosystems) listed below:

Gene Name	Assay ID
ACHE	Hs00241307_m1
NES	Hs00707120_s1
TUBB3	Hs00964962_g1
GFAP	Hs00157674_m1
PAX6	Hs00240871_m1
MSI1	Hs01045894_m1
NGN2	Hs00702774_s1
MAP2	Hs00258900_m1
GAPDH (housekeeping gene)	Hs99999905_m1
PPIA (housekeeping gene)	Hs99999904_m1

The FAST 96-well reaction was performed with 8 ng cDNA per well in a 10 μl reaction with 40 cycles. Thermal cycler conditions were as follows: 20 seconds at 95° C., and 1 second at 95° C., 20 seconds at 60° C. for 40 cycles.

Relative Expression values were calculated as previously described in Example I, except the Average of 2 Housekeeping genes (GAPDH & PPIA) was used for normalization instead of the Average of 3 Housekeeping genes. Identification of neuronal lineage genes was investigated following the transfection with three independent vectors containing Msi1, Ngn2, and Pax6.

As shown in Table 2, after 14 days following transfection, relative expression of mRNA of neuronal lineage was undetectable in untransfected cells (HFF), while the cells transfected with Msi1 or Ngn2 in the presence of MBD2 expressed neural stem cell markers (Nestin and Sox2), however the expression of Sox2 was much more highly expressed than nestin following transfection with Ngn2 or Msi1. Neuronal and astrocyte specific genes (βIII-Tubulin, MAP2b, GFAP, and ACHE) was increased as well. mRNAs level of the tripotent-associated genes 131H-tubulin, MAP2b, acetycholine, and GFAP were undetectable in Pax6 transfected cells, indicating that Pax6 alone was not implicated in the reprogramming process toward neuronal lineage.

TABLE 2

Relative expression of gene expression of different neuronal lineage performed by RT-PCR following the transfection of HFF by Msi1, Ngn2, or Pax6 in the presence of MBD2 and cultured for 14 days.

	MSI1		NGN2		PAX6		NES		TUBB3	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1 Control Untransfect.	1.00	0.07	1.08	0.57	1.11	0.67	1.00	0.02	1.00	0.01
#2 MSI1/MBD2	4077.82	248.02	1.18	0.66	487.09	69.58	8.62	0.00	6.58	0.11
#3 NGN2/MBD2	14.16	0.63	47803.26	192.78	624.31	91.27	8.62	0.02	8.33	0.02
#4 PAX6/MBD2	1.70	0.36	0.27	0.01	29564.43	357.89	0.46	0.00	0.49	0.02

	ACHE		GFAP		MAP2		SOX2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1 Control Untransfect.	1.02	0.29	1.00	0.06	1.00	0.01	1.00	0.09
#2 MSI1/MBD2	6.58	0.64	215.71	20.65	5.50	0.46	3499.53	184.85
#3 NGN2/MBD2	8.33	0.97	365.60	5.11	5.42	0.00	4039.03	8.65
#4 PAX6/MBD2	1.98	0.48	1.15	0.13	0.55	0.04	1.00	0.03

Immunohistochemical Analysis

Fluorescent immunohistochemical staining was performed as previously described in Example I. In agreement with the RT-PCR data, immunohistochemical analysis of these cultures revealed that reprogrammed cells (with Msi1 or Ngn2) generated morphologically complex neurons that were positive for MAP2b, indicating the differentiation of NSLCs to neuron-like cells (NLCs). However, the positive staining for these markers was undetectable after transfection with Pax6/MBD2. Moreover, the newly formed neurons expressed the markers for and developed long neurites with growth cones at their ends, expressed neural specific genes, and ceased to proliferate when they were exposed to differentiation conditions.

Example III

Transfection of HFF by Various Combinations of Vectors and Disruption of Cell Cytoskeleton

Various combinations of neurogenic regulators and cytokines for epigenetic modifications were tested to ascertain their effect on reprogramming efficiency. Starting one day before transfection, cells were treated with or without cytochalasin B (Calbiochem), with the concentration decreased every day over five days during media changes (starting with 10 μg/ml Cytochalasin B on day 1 to 7.5 μg/ml, 5 μg/ml, 2.5 μg/ml, and 0 μg/ml over the subsequent four days) in order to investigate the effect of disrupting the cell cytoskeleton on the process of reprogramming. Cells were transiently transfected as described in Example II with one or two vectors containing one neurogenic transcription factors by nucleofection. Cells were co-transfected with either of two DNA demethylators, MBD2 or GAdd45B, (e.g. 2×10^6 cells were transfected with pCMV6-XL5Msi1 (2 μg) and pCMV6-XL5-MBD2 (2 μg)). After 24 hours, the medium was changed to Neural Proliferation Medium (NeuroCult™ proliferation Kit, StemCell Technologies) consisting of DMEM/F12 (1:1), glucose (0.6%), sodium bicarbonate (0.1%), glutamine (20 mM), HEPES (5 mM), insulin (230 μg/ml), transferrin (100 μg/ml), progesterone (200 nM), putrescine (90 μg/ml), and sodium selenite (300 nM) and supplemented with Noggin (20 ng/ml, Peprotech), recombinant hFGF (20 ng/ml, Peprotech), and recombinant hEGF (20 ng/ml, Peprotech) and cells were cultured for two weeks at 37° C., 5% CO_2 and 5% O_2. Cells were then analyzed for neural stem cell markers.

Gene Expression Analysis

Gene expression analysis was performed for neural stem-specific markers (Sox2, Nestin, GFAP) and a fibroblast-specific marker (Col5A2) by RT-PCR as previously described in Example I. RT-PCR analysis showed that the relative expression of Sox2, nestin and GFAP was enhanced after transfecting the cells with the neurogenic transcription factors. As shown in Table 3, transfecting the cells with one transcription factor Msi1 in the presence of Gadd45b was associated with up-regulation of relative expression of Sox2 (22.3±5.26) and GFAP (10.14±0.15) and the expression of the these genes was highly increase when transfecting the cells with Ngn2 by 20 fold and 10 fold respectively. Combining the two neurogenic factors (Msi1 and Ngn2) with Gadd45b enhanced further the expression of Sox2 and GFAP. Transfecting the cells with one transcription factor (Msi1 or Ngn2) in the presence of MBD2 was associated with up-regulation of relative expression of Sox2, Nestin, and GFAP and down-regulation of Col5A2, while co-transfection with Gadd45b did not increased the expression of nestin and the expression of Col5A2 was not regulated. The enhancement of neural stem cells relative expression was observed when transfecting the cells with two neurogenic genes in combination with MBD2; a small increase in the expression was noticed in the presence of cytochalasin B under certain conditions. An increase in the relative expression of the neural stem-specific markers (Sox2, Nestin, GFAP) and a decrease in the fibroblast-specific gene (COL5A2) was observed after transfection with Msi1/Ngn2/MBD2, Msi1/Ngn2/Gadd45b, Msi1/MBD2 or Ngn2/MBD2 (Table 3). This study demonstrated that MBD2 increased more reprogramming efficiency then GDA45b and showed that cytochalasin B had no effect of its own in the control cultures.

TABLE 3

RT-PCR analysis of relative expression of neuronal precursor cell markers such as nestin, Sox2, and GFAP after transfection of fibroblast cells with different combinations with or without the co-treatment with cytochalasin B. Relative expression of Sox2, nestin, and GFAP in NSLCs was increased after transfection with both transcription factors (Ngn2 and Msi1) with MBD2 as the DNA demethyaltor. As demonstrated, this upregulation of neural stem cell gene expression was associated with a decrease of CoL5A2, a specific gene for fibroblast cells.

	COL5A2		FBN2		NES		MAP2		TUBB3		SOX2		ACHE		GFAP	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1, +CytoB, Control	1.00	0.07	1.00	0.01	1.00	0.04	1.00	0.05	1.00	0.05	1.00	0.05	1.00	0.10	1.00	0.11
#2, −CytoB, Control	1.00	0.03	1.00	0.08	1.00	0.00	1.00	0.09	1.00	0.09	1.15	0.80	1.01	0.18	1.00	0.01
#3, +CytoB, Msi1, GAD45b	0.85	0.04	0.75	0.02	0.60	0.01	0.29	0.01	0.44	0.00	22.39	5.26	0.81	0.19	10.14	0.15
#4, −CytoB, Msi1, GAD45b	0.87	0.03	1.81	0.09	1.84	0.04	2.31	0.00	2.09	0.03	20.28	5.33	1.99	0.74	6.03	0.05
#5, +CytoB, Ngn2, GAD45b	0.84	0.04	0.77	0.03	0.44	0.00	0.24	0.00	0.36	0.01	470.84	13.43	0.63	0.05	103.22	0.80
#6, −CytoB, Ngn2, GAD45b	0.75	0.07	1.97	0.02	1.83	0.00	4.40	0.16	2.02	0.10	789.33	60.35	1.70	0.13	110.48	4.90
#7, +CytoB, Pax6, GAD45b	0.74	0.12	1.08	0.00	0.89	0.01	0.51	0.00	0.63	0.04	1.64	0.98	0.86	0.12	2.49	0.21
#8, −CytoB, Pax6, GAD45b	0.66	0.04	2.41	0.09	2.70	0.03	4.96	0.30	3.48	0.07	0.46	0.33	2.97	1.04	0.43	0.09
#9, +CytoB, Msi1, Ngn2, GAD45b	0.14	0.01	0.28	0.01	1.30	0.03	4.07	0.11	0.84	0.00	54768.27	6709.56	0.81	0.24	3391.96	64.63
#10, −CytoB, Msi1, Ngn2 GAD45b	0.12	0.00	0.73	0.03	5.28	0.21	50.84	1.23	4.93	0.28	17400.66	822.88	3.58	0.10	1255.76	5.27
#11, +CytoB, Msi1, Ngn2 MBD2	0.10	0.00	0.26	0.01	1.11	0.01	3.69	0.09	0.76	0.00	55588.41	1331.20	0.55	0.14	2849.96	261.51
#12, −CytoB, Msi1, Ngn2 MBD2	0.44	0.01	1.47	0.06	5.49	0.14	47.30	0.11	5.50	0.31	14587.46	789.19	3.90	0.13	1424.04	39.29

TABLE 3-continued

RT-PCR analysis of relative expression of neuronal precursor cell markers such as nestin, Sox2, and GFAP after transfection of fibroblast cells with different combinations with or without the co-treatment with cytochalasin B. Relative expression of Sox2, nestin, and GFAP in NSLCs was increased after transfection with both transcription factors (Ngn2 and Msi1) with MBD2 as the DNA demethyaltor. As demonstrated, this upregulation of neural stem cell gene expression was associated with a decrease of CoL5A2, a specific gene for fibroblast cells.

	COL5A2		FBN2		NES		MAP2		TUBB3		SOX2		ACHE		GFAP	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#13, +CytoB, GAD45b	1.11	0.04	1.09	0.06	0.92	0.08	0.68	0.01	0.82	0.03	63.93	2.81	1.19	0.17	17.43	1.86
#14, −CytoB, GAD45b	0.94	0.01	2.22	0.00	2.82	0.02	6.49	0.30	4.01	0.05	6.12	0.61	2.34	0.17	1.42	0.10
#15, +CytoB, MBD2	0.83	0.00	0.83	0.05	0.36	0.01	0.16	0.01	0.36	0.00	3.42	3.74	0.63	0.37	2.18	0.12
#16, −CytoB, MBD2	0.68	0.02	1.55	0.04	1.57	0.05	1.47	0.01	2.00	0.00	0.52	0.29	1.45	0.15	0.55	0.04
#17, +CytoB, Msi1, Ngn2	1.10	0.01	1.16	0.03	1.37	0.01	1.12	0.06	0.86	0.06	5.59	1.48	1.07	0.27	1.70	0.46
#18, −CytoB, Msi1, Ngn2	0.93	0.04	2.52	0.10	3.48	0.01	9.01	0.02	4.55	0.18	1.78	1.46	3.83	0.42	0.59	0.01
#19, +CytoB, Msi1, MBD2	0.20	0.03	0.36	0.01	1.25	0.05	6.68	0.31	0.72	0.02	66592.29	3481.89	2.57	0.03	4450.08	131.85
#20, −CytoB, Msi1, MBD2	0.12	0.00	0.64	0.03	4.70	0.22	77.51	0.11	4.12	0.11	19128.03	1542.00	8.14	0.13	999.22	24.75
#21, +CytoB, Ngn2, MBD2	0.17	0.01	0.28	0.00	1.16	0.04	5.73	0.06	0.62	0.00	67945.51	3000.74	2.15	0.04	4736.83	11.92
#22, −CytoB, Ngn2, MBD2	0.17	0.00	0.78	0.03	4.32	0.08	68.89	5.26	4.01	0.04	16570.91	92.96	7.04	0.53	1427.13	13.19
#23, +CytoB, Msi1	0.71	0.05	0.79	0.06	0.87	0.01	0.63	0.06	0.67	0.04	2.86	0.70	1.08	0.08	2.08	0.11
#24, −CytoB, Msi1	0.66	0.04	1.92	0.17	2.03	0.02	2.77	0.02	2.68	0.02	0.32	0.12	1.85	0.65	0.58	0.04

Immunohistochemical Analysis

Fluorescent immunohistochemical staining was performed as previously described in Example I. Table 4 shows the percentage of Nestin and Sox2 in each condition, with the highest percentage of Sox2 (38.18±1.75%) and nestin (28.18±2.77%) positive cells observed after transfecting the cells simultaneously with both neurogenic transcription factors and in the presence of a DNA demethylator and cytochalasin B. A slight increase of Sox2 positive cells (10.42±10.27%) and nestin positive cells (4.85±1.10%) was detected following transfection with one transcription factor Msi1 and MBD2. Same tendency of nestin and Sox2 positive cells was observed following transfection with Ngn2 and MBD2. Disrupting the cell cytoskeleton with Cytochalasin B significantly enhanced reprogramming, but had no reprogramming effect on its own (Table 4).

TABLE 4

Percentage of positive cells for Sox2 and nestin after transfection of fibroblast cells with different expression vectors with or without the presence of cytochalasin B. After transfection the cells were cultured in proliferation medium (StemCell Technologies) supplemented by EGF (20 ng/ml, Peprotech) and FGF (20 ng/ml, Peprotech) for two weeks at 37° C./5% CO_2/5% O_2. The percentage of immunopositive cells was determined by Cellomics ™ and represented as mean ± SD (n = 3-5).

	% of Sox2 positive cells		% of Nestin positive cells	
	+CytoB	−Cyto B	+CytoB	−CytoB
Untransfected cells	0.02 ± 0.01	0.01 ± 0.00	0.14 ± 0.04	0.11 ± 0.09
Ngn2	0.35 ± 0.36	0.15 ± 0.05	2.34 ± 0.99	1.04 ± 0.21
Msi1	0.23 ± 0.15	0.12 ± 0.09	1.95 ± 0.11	1.11 ± 0.18
Gadd45b	0.30 ± 0.17	0.29 ± 0.11	4.94 ± 0.25	2.33 ± 0.42
MBD2	0.22 ± 0.13	0.22 ± 0.11	2.8 ± 0.11	1.53 ± 0.6
Msi1/Ngn2	0.19 ± 0.13	0.32 ± 0.05	1.91 ± 0.56	2.59 ± 1.28

TABLE 4-continued

Percentage of positive cells for Sox2 and nestin after transfection of fibroblast cells with different expression vectors with or without the presence of cytochalasin B. After transfection the cells were cultured in proliferation medium (StemCell Technologies) supplemented by EGF (20 ng/ml, Peprotech) and FGF (20 ng/ml, Peprotech) for two weeks at 37° C./5% CO_2/5% O_2. The percentage of immunopositive cells was determined by Cellomics ™ and represented as mean ± SD (n = 3-5).

	% of Sox2 positive cells		% of Nestin positive cells	
	+CytoB	−Cyto B	+CytoB	−CytoB
Msi1/MBD2	10.42 ± 10.27	8.84 ± 11.63	4.85 ± 1.10	2.06 ± 0.08
Msi1/Gadd45b	0.06 ± 0.01	0.14 ± 0.17	0.55 ± 0.06	0.24 ± 0.11
Ngn2/MBD2	11.17 ± 0.08	9.07 ± 11.31	5.7 ± 0.10	2.18 ± 0.23
Ngn2/GAdd45b	0.29 ± 0.11	0.95 ± 0.17	1.17 ± 0.54	0.98 ± 0.25
Msi1/Ngn2/MBD2	38.18 ± 1.75	22.03 ± 1.90	28.18 ± 2.77	14.54 ± 0.45
Msi1/Ngn2/Gadd45b	22.65 ± 5.03	18.54 ± 9.40	18.72 ± 6.26	8.70 ± 4.51

Various DNA demethylators were tested as well for their effect on reprogramming efficiency. Cells were co-transfected with one vector (MSI1/NGN2) containing two neurogenic pCMV6-Msi1-Ngn2 factors with various DNA demethylators. Simultaneously another neurogenic factor was tested for its effect on cells de-differentiation towards NSCs, pCMV-XL-Nestin individually or in combination with pCMV-Msi1-Ngn2, pCMV-XL5-Msi1, or pCMV-XL4-Ngn2 in the presence of MBD2 as previously described in Example II.

Cells were co-transfected pCMV-Msi1-Ngn2 with different DNA demethylators (MBD1, MBD2, MBD3, MBD4, MeCP2, AICDA). Another assay was performed to assess the effect of nestin on the reprogramming efficiency; therefore cells were transfected with nestin individually or in combination with one vector containing one neurogenic factor (Msi1 or Ngn2) or both neurogenic factors in the presence of MBD2. Cells were cultured following transfection in the presence of proliferation medium supplemented with EGF (20 ng/ml), FGF (20 ng/ml), and Noggin (20 ng/ml) with and without VPA (1 mM) treatment for 12 days at 37° C., 5% CO_2 and 5% O_2.

Gene expression analysis and immunohistochemistry was performed to analyse neural specific gene and protein expression (βIII-tubulin, GFAP, Sox2, Nestin) as described in Example II. Transfecting cells with Msi1 and Ngn2 in the presence of various DNA demethylators revealed and confirm previous data showing that the among various DNA demethylators used in this study, MBD2 promotes the expression of neural stem genes (Sox2, GFAP, Nestin) as shown in Table 5. Furthermore, transfecting cells with nestin with and without the presence of one neurogenic factor had no effect on the reprogramming efficiency into neural stem-like cells. However co-transfection with nestin and Msi1/Ngn2/MBD2 enhanced the expression of neural stem cells genes and this increase was more pronounced in the presence of VPA.

TABLE 5

RT-PCR analysis of relative expression of neuronal precursor cell markers such as nestin, Sox2, βIII-tubulin, and GFAP after transfection of fibroblast cells with various combinations of pCMV-Msi1-Ngn2 (MSI1/NGN2), pCMV-XL5-Msi1, pCMV-XL4-Ngn2, pCMV-XL-Nestin with different combinations of DNA demethylators, with and without the co-treatment with VPA.

	TUBB3		GFAP		SOX2		NES	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
Day 12, Untransfected (−VPA)	1.00	0.11	1.00	0.05	1.01	0.16	1.00	0.13
Day 12, Untransfected (+VPA)	1.00	0.03	1.00	0.06	1.00	0.00	1.00	0.02
Day 12, MSI1/NGN2/MBD1 (−VPA)	0.96	0.06	2.69	0.13	1.15	0.49	0.46	0.02
Day 12, MSI1/NGN2/MBD1 (+VPA)	1.10	0.06	2.22	0.06	0.80	0.01	0.84	0.02
Day 12, MSI1/NGN2/MBD2 (−VPA)	123.52	0.06	1638.53	99.86	61467.29	1487.21	31.77	0.17
Day 12, MSI1/NGN2/MBD2 (+VPA)	232.00	0.08	1889.30	42.39	72022.15	7894.41	42.69	0.14
Day 12, MSI1/NGN2/MBD3 (−VPA)	0.92	0.07	3.98	0.59	28.05	4.67	0.56	0.01
Day 12, MSI1/NGN2/MBD3 (+VPA)	1.23	0.05	1.66	0.18	11.31	2.35	0.87	0.02
Day 12, MSI1/NGN2/MBD4 (−VPA)	0.85	0.01	4.80	0.23	5.42	5.20	0.62	0.00
Day 12, MSI1/NGN2/MBD4 (+VPA)	0.95	0.01	1.57	0.16	2.27	0.04	0.79	0.03
Day 12, MSI1/NGN2/MeCP2 (−VPA)	1.11	0.06	3.80	0.38	6.54	6.45	0.69	0.01
Day 12, MSI1/NGN2/MeCP2 (+VPA)	1.37	0.09	1.63	0.45	10.53	10.49	1.07	0.01
Day 12, MSI1/NGN2/AICDA (−VPA)	1.07	0.04	4.59	0.02	0.65	0.01	0.74	0.02
Day 12, MSI1/NGN2/AICDA (+VPA)	1.10	0.01	2.37	0.29	1.21	0.16	0.91	0.04
Day 12, Msi1/MBD2 (−VPA)	1.31	0.17	3.78	0.49	0.70	0.02	0.78	0.00
Day 12, Msi1/MBD2 (+VPA)	1.36	0.07	1.75	0.31	1.26	0.03	1.15	0.03
Day 12, Ngn2/MBD2 (−VPA)	0.85	0.06	2.93	0.51	0.79	0.05	0.58	0.02
Day 12, Ngn2/MBD2 (+VPA)	1.41	0.05	1.60	0.11	2.30	0.06	1.03	0.03
Day 12, Nes/Msi1 (−VPA)	0.84	0.03	3.21	0.72	0.76	0.01	0.51	0.01
Day 12, Nes/Msi1 (+VPA)	0.86	0.09	1.82	0.30	2.14	1.02	0.94	0.01
Day 12, Nes/Ngn2 (−VPA)	0.69	0.05	2.88	0.32	0.99	0.10	0.57	0.02
Day 12, Nes/Ngn2 (+VPA)	0.88	0.01	1.53	0.19	2.71	0.02	0.83	0.03
Day 12, Nes/MSI1/NGN2/MBD2 (−VPA)	111.58	0.04	1423.56	82.87	72069.27	624.51	51.52	0.12
Day 12, Nes/MSI1/NGN2/MBD2 (+VPA)	321.00	0.04	2600.14	1.90	88932.00	708.72	82.74	0.18
Day 12, Nes/MSI1/NGN2 (−VPA)	0.74	0.11	2.60	0.28	1.98	0.97	0.55	0.01
Day 12, Nes/MSI1/NGN2 (+VPA)	0.86	0.00	1.70	0.49	1.70	0.04	0.88	0.05
Day 12, Nes/MBD2 (−VPA)	0.76	0.12	3.15	0.17	0.87	0.03	0.44	0.00
Day 12, Nes/MBD2 (+VPA)	0.87	0.03	2.05	0.07	2.66	1.64	0.91	0.00
Day 12, Nes/Msi1/MBD2 (−VPA)	0.81	0.05	3.41	0.66	1.11	0.01	0.58	0.01
Day 12, Nes/Msi1/MBD2 (+VPA)	1.01	0.13	2.43	0.07	3.27	0.26	0.93	0.02
Day 12, Nes/Ngn2/MBD2 (−VPA)	1.19	0.07	5.71	1.30	4.11	0.07	0.91	0.04
Day 12, Nes/Ngn2/MBD2 (+VPA)	1.29	0.03	2.98	0.66	21.20	0.42	1.65	0.02

Immunohistochemistry analysis performed in parallel with RT-PCR data indicated that positive Sox2 cells were undetectable when transfecting the cells with Msi1/Ngn2 in the presence of MBD1, MBD3, MBD4, MeCP1, or AICADA (Table 6) and that among the different types of DNA demethylator genes tested only MBD2 plays a significant positive role in the reprogramming efficiency of HFF towards NSLCs when using the above neurogenic genes. Immunohistochemistry analysis revealed a small increase of immunopositive Sox2 cells (89.49±3.18) after co-transfecting the cells with nestin and Msi1/Ngn2 in the presence of MBD2 (Table 6).

TABLE 6

Percentage of positive cells for Sox2 after transfection of fibroblast cells with different expression vectors with or without the presence of various DNA demethylators. After transfection the cells were cultured in proliferation medium (StemCell Technologies) supplemented by EGF (20 ng/ml, Peprotech) and FGF (20 ng/ml, Peprotech) for two weeks at 37° C./5% CO_2/5% O_2. The percentage of immunopositive cells was determined by Cellomics ™ and represented as mean ± SD (n = 3-5).

	% Sox2 positive ± stdv
HFF untransfected	0.13 ± 0.12
Msi-Ngn2 + MBD1	0.92 ± 0.13
Msi-Ngn2 + MBD2	79.44 ± 9.86
Msi-Ngn2 + MBD3	1.22 ± 0.82
Msi-Ngn2 + MBD4	0.59 ± 0.03
Msi-Ngn2 + MeCP2	1.10 ± 0.25
Msi-Ngn2 + AICDA	0.69 ± 0.28
Msi + MBD2	0.79 ± 0.28
Ngn2 + MBD2	1.74 ± 1.01
Nestin + Msi	0.91 ± 0.01
Nestin + Ngn2	2.16 ± 1.44
Nestin + MSI1/NGN2 + MBD2	89.49 ± 3.18
Nestin + MSI1/NGN2	10.20 ± 0.21
Nestin + MBD2	0.00 ± 0.00
Nestin + Msi + MBD2	8.45 ± 0.08
Nestin + Ngn2 + MBD2	5.71 ± 0.66

Another study was designed to test the effect of various neurogenic genes on the reprogramming efficiency towards neural stem-like cells. HFF cells were cultured as described in Example I, and transfected using the Nucleofector™ 96-well Shuttle Device (Lonza) following procedure described in Example IV, except for the untreated HFF control and the untransfected HFF control (for determining the effect of the complete media & compound treatments on the cells). The cells that had been pre-treated with VPA and 5-Aza and the untreated cells were transfected with the mixes of DNA as described in Table 7. The cells were plated on Laminin-coated plates and incubated at 37° C., 5% CO_2. Media was changed daily according to Table 7. Cells were analysed at day 3, 7, 12 by immunohistochemistry analysis and at Day 9 by gene array for multipotent and pluripotent gene expression.

Gene Array Analysis

An additional batch of cells treated according to 0a and 1a in Table 7 was analyzed at Day 9, along with HFFs, hNPCs, and passage 5 NSLCs (frozen from previous experiments from Example III) by the Pluripotency Gene Array (ABI) (Tables 8a and b) and a set of genes (Table 8c) to determine the gene expression profile of select pluripotency, ectoderm, endoderm, mesoderm, and neural lineage genes in passage 1 and passage 5 NSLCs compared to HFFs (from which they were created) and normal human neuroprogenitor cells (hNPCs). The results in Table 8 indicate that all the genes related to neural stem cells (some of the significantly expressed pluripotency markers and mesendoderm markers are also expressed in neural stem cells) and the neural lineage were significantly expressed in NSLCs as opposed to HFFs, and the expression pattern was a bit different from hNPCs indicating that NSLCs are similar to, but not identical, to the hNPCs tested. Passage 5 NSLCs 5 had a higher expression of stemness genes than Passage 1 NSLCs, hNPCs had a higher expression of neuronally committed genes than NSLCs, indicting their neuroprogenitor status versus the greater stemness status of NSLCs.

TABLE 7

Plasmids and media composition from day 1 to day 12.

	From day −2 to day 0	Plasmids transfected at day 0	From day 1 to day 3	From day 3 to day 4	From day 4 to day 12
0a	Untreated	No plasmid	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
1a	Untreated	Msi1/Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
1b	Untreated	Msi1/Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2 + SHH
1c	Untreated	Msi1/Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin (day 1 to day 7)/Forskolin (day 7 to day 12)
1d	Untreated	Msi1/Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
1e	Untreated	Msi1/Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
1f	Untreated	Msi1/Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
2	Untreated	Msi1/Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2 + CytoB	Neural proliferation medium + Egf + Fgf-2 + CytoB	Neural proliferation medium + Egf + Fgf-2
3	Untreated	Msi1/Ngn2	Neural proliferation medium + Egf + Fgf-2 + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
4	Untreated	Msi1/Ngn2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2

TABLE 7-continued

	Plasmids and media composition from day 1 to day 12.				
	From day −2 to day 0	Plasmids transfected at day 0	From day 1 to day 3	From day 3 to day 4	From day 4 to day 12
5	Untreated	pCMV6-XL5-Musashi	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
6	Untreated	pCMV6-XL5-Musashi	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin + Forskolin
7	Untreated	pCMV6-XL5-Musashi	Neural proliferation medium + Egf + Fgf-2 + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
8	Untreated	pCMV6-XL5-Musashi	Neural proliferation medium + Egf + Fgf-2 + Noggin + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin + Forskolin
9	Untreated	pCMV6-XL5-ZIC1 + pCMV6-XL4-Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
10	Untreated	pCMV6-XL5-SOX1 + pCMV6-XL4-Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
11	Untreated	pCMV6-XL5-Sox2 + pCMV6-XL4-Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
12	Untreated	pCMV6-XL5-Nanog + pCMV6-XL4-Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
13	Untreated	pCMV6-XL4-Oct4 + pCMV6-XL4-Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
14	VPA + 5-Aza pre-treated	Msi1/Ngn2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
15	VPA + 5-Aza pre-treated	pCMV6-XL5-Musashi	Neural proliferation medium + Egf + Fgf-2 + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2	Neural proliferation medium + Egf + Fgf-2
16	VPA + 5-Aza pre-treated	pCMV6-XL5-Musashi	Neural proliferation medium + Egf + Fgf-2 + Noggin + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin + Forskolin
17	VPA + 5-Aza pre-treated	pCMV6-XL5-Musashi + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2 + Noggin + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin + Forskolin
18	VPA + 5-Aza pre-treated	pCMV6-XL4-Ngn2	Neural proliferation medium + Egf + Fgf-2 + Noggin + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin + Forskolin
19	VPA + 5-Aza pre-treated	pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2 + Noggin + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin + Forskolin
20	VPA + 5-Aza pre-treated	Ngn2 + pCMV6-XL5-MBD2	Neural proliferation medium + Egf + Fgf-2 + Noggin + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin + Forskolin
21	VPA + 5-Aza pre-treated	No plasmid	Neural proliferation medium + Egf + Fgf-2 + Noggin + VPA + 5-Aza	Neural proliferation medium + Egf + Fgf-2 + Noggin	Neural proliferation medium + Egf + Fgf-2 + Noggin + Forskolin

* Immunohistochemistry analysis performed in parallel with RT-PCR data indicated among all the combinations in this experiment where no cytochalasin B was used, positive Sox2 cells were detectable only in cells transfected with Msi1/Ngn2 with and without MBD2.

TABLE 8a

Results for Human Stem Cell Pluripotency Array (n = 4 for each sample) - Embryonic Stem Cell Markers, Germ Cell Markers and Trophoblast Markers.

Gene	Untreated HFF (Passage 8) Rel. Exp.	Std. Dev.	Untransfected HFF (Day 9) Rel. Exp.	Std. Dev.	hNPC neurospheres (Passage 4) Rel. Exp.	Std. Dev.	MSI1/NGN2/MBD2-transfected HFF (Day 9) (NSLC, Passage 1) Rel. Exp.	Std. Dev.	Neural stem-like cells, NSLC (Passage 5) Rel. Exp.	Std. Dev.
Embryonic Stem cell markers										
BRIX	1.03	0.30	0.47	0.10	0.78	0.22	0.78	0.25	0.83	0.10
CD9	1.01	0.18	2.46*	0.62	1.86	0.29	2.24*	0.19	1.00	0.39
COMMD3	1.08	0.53	0.94	0.36	0.94	0.40	0.98	0.40	1.05	0.59
DNMT3B	1.07	0.50	0.34*	0.14	2.96*	0.84	1.90	0.41	0.35	0.34

TABLE 8a-continued

Results for Human Stem Cell Pluripotency Array (n = 4 for each sample) - Embryonic Stem Cell Markers, Germ Cell Markers and Trophoblast Markers.

Gene	Untreated HFF (Passage 8) Rel. Exp.	Std. Dev.	Untransfected HFF (Day 9) Rel. Exp.	Std. Dev.	hNPC neurospheres (Passage 4) Rel. Exp.	Std. Dev.	MSI1/NGN2/MBD2-transfected HFF (Day 9) (NSLC, Passage 1) Rel. Exp.	Std. Dev.	Neural stem-like cells, NSLC (Passage 5) Rel. Exp.	Std. Dev.
EBAF/LEFTY2	1.00	0.00	2.10	0.00	7.95	4.60	7.79	4.88	70.56*	26.12
FGF4	1.00	0.00	2.10	0.00	1.44	0.00	1.54	0.00	1.37	0.00
FOXD3	1.00	0.00	2.10	0.00	1.44	0.00	7.13	11.18	222.41*	63.43
GABRB3	1.06	0.38	4.22*	0.71	66.65*	12.52	40.01*	4.54	1.62	0.98
GAL	1.00	0.04	9.73*	0.32	0.03*	0.01	4.25*	0.46	2.89*	0.83
GBX2	1.00	0.09	0.04	0.05	45.28*	4.59	90.92*	12.14	55.22*	2.36
GDF3	1.00	0.00	2.10	0.00	1.44	0.00	1.54	0.00	1.37	0.00
GRB7	1.02	0.24	0.30*	0.16	0.05*	0.04	0.29*	0.08	0.06*	0.08
IFITM1	1.01	0.17	63.96*	6.04	0.04*	0.01	21.80*	4.31	3.35*	0.63
IFITM2	1.00	0.12	3.84*	0.89	0.02*	0.00	0.65	0.11	0.43*	0.09
IL6ST	1.01	0.21	2.19*	0.39	0.85	0.14	1.59	0.26	0.75	0.06
IMP2	1.11	0.66	1.65	0.92	1.06	0.48	0.78	0.26	1.96	0.97
KIT	1.02	0.26	1.15	0.30	0.02*	0.00	0.31*	0.09	0.00*	0.00
LEFTB	1.61	1.15	12.28*	7.84	5.45	3.15	5.58	2.65	8.96*	4.12
LIFR	2.29	3.57	13.51	16.55	6.31	7.24	12.98	9.81	2.85	4.31
LIN28	4.69	8.62	5.25	8.88	28.38*	19.25	26.97*	8.68	32.13*	14.32
NANOG	1.71	1.97	18.61	16.43	64.94*	28.32	70.87*	9.88	5.87	3.52
NOG	1.03	0.27	0.18*	0.08	0.18*	0.06	0.22*	0.06	0.02*	0.00
NR5A2	2.04	2.05	6.85	8.80	0.38	0.00	3.89	4.36	0.36	0.00
NR6A1	1.11	0.66	1.37	0.31	5.08*	0.37	2.71*	0.63	2.04*	0.17
PODXL	1.00	0.07	0.01*	0.01	0.80	0.11	2.09*	0.04	6.49*	0.64
POU5F1	1.01	0.13	0.27*	0.17	0.89	0.09	0.71	0.09	0.19*	0.06
PTEN	1.00	0.02	2.68*	0.29	0.87	0.04	1.07	0.12	0.80	0.14
RESET	1.01	0.12	1.53	0.17	0.94	0.18	1.04	0.21	1.10	0.24
SEMA3A	1.00	0.11	1.99	0.19	0.66	0.05	1.05	0.11	0.90	0.16
SFRP2	1.11	0.56	122.57*	14.57	3480.98*	702.37	1500.84*	272.46	2.75	2.85
SOX2	1.00	0.00	2.45	0.70	127594.46*	11326.91	88615.76*	15003.70	137424.37*	26622.02
TDGF1	1.41	1.28	2.92	0.68	6.13	1.52	5.46	1.95	2.20	1.51
TERT	1.00	0.00	2.10	0.00	10.81	18.75	10.74	18.41	6506.88*	893.84
TFCP2L1	1.00	0.00	2.10	0.00	7.84	12.80	32.49	10.01	1.37	0.00
UTF1	1.00	0.00	8.21	12.23	27.86	19.24	1.54	0.00	30.68	25.94
XIST	1.00	0.00	2.10	0.00	24609.46*	4337.83	22637.95*	3988.10	1.37	0.00
ZFP42	1.24	1.06	12.38	12.58	1.41	0.78	2.01	1.85	1.76	0.93
Germ cell markers										
DDX4	1.00	0.00	2.10	0.00	1.44	0.00	5.84	8.60	19.11	20.49
SYCP3	1.58	1.95	11.97	8.01	11.12	3.46	15.46	11.65	2.25	2.85
Trophoblast markers										
CDX2	1.00	0.00	2.10	0.00	1.44	0.00	1.54	0.00	1.37	0.00
CGB	1.02	0.24	2.08*	0.74	0.15*	0.16	0.57	0.41	0.09*	0.17
EOMES	1.51	1.14	0.33	0.00	0.71	0.97	0.24	0.00	0.77	1.12
GCM1	2.61	2.80	0.42	0.00	3.25	5.92	5.68	1.44	1.47	2.38
KRT1	1.00	0.00	2.10	0.00	1.44	0.00	1.54	0.00	1.37	0.00

For Relative Expression calculations, each sample was normalized to the average Ct of the 6 housekeeping genes (ACTB, 18S, CTNNB1, EEF1A1, GAPD, RAF1), and calibrated to the Untreated HFF (Passage 8) control.

Relative Expression values with asterisk (*) indicate values with significant up or down-regulation (>2-fold or <0.5-fold). For these samples, for Ct values <35 is considered that the expression of the gene is adequate for quantification.

For the Relative Expression values that are >2-fold or <0.5-fold but without asterisk, the values could have significant error due to the low expression of the gene (Ct > 35), and thus the up or down-regulation could be merely a result of the high standard deviation of the high Ct values of the genes, or fluctuations in the housekeeping genes.

As for the Relative Expression values that are between 0.5-fold and 2-fold, it indicates no significant change in the expression of the gene for these samples.

TABLE 8b

Results for Human Stem Cell Pluripotency Array (n = 4 for each sample) - Ectoderm, Endoderm and Mesoderm Markers.

Gene	Untreated HFF (Passage 8) Rel. Exp.	Untreated HFF (Passage 8) Std. Dev.	Untransfected HFF (Day 9) Rel. Exp.	Untransfected HFF (Day 9) Std. Dev.	hNPC neurospheres (Passage 4) Rel. Exp.	hNPC neurospheres (Passage 4) Std. Dev.	MSI1/NGN2/MBD2-transfected HFF (Day 9) (NSLC, Passage 1) Rel. Exp.	MSI1/NGN2/MBD2-transfected HFF (Day 9) (NSLC, Passage 1) Std. Dev.	Neural stem-like cells, NSLC (Passage 5) Rel. Exp.	Neural stem-like cells, NSLC (Passage 5) Std. Dev.
Ectoderm markers										
CRABP2	1.04	0.35	26.14*	4.28	0.01*	0.01	21.11*	2.80	0.21*	0.05
FGF5	1.01	0.15	0.21*	0.07	0.00*	0.00	0.10*	0.02	0.00*	0.00
GFAP	1.22	0.84	9.89*	5.46	798.04*	162.37	487.99*	79.84	12052.09*	2984.71
ISL1	1.01	0.12	2.19*	0.27	0.02*	0.02	0.42*	0.08	0.00*	0.00
NES	1.10	0.58	3.19*	0.95	6.78*	0.95	3.84*	0.19	7.47*	0.54
NEUROD1	1.00	0.00	2.10	0.00	1.44	0.00	2.32	1.57	25.54	6.42
OLIG2	1.00	0.00	2.10	0.00	124181.50*	14735.13	80826.42*	27820.32	36172.45*	3145.67
PAX6	1.11	0.48	0.06*	0.00	533.31*	120.59	326.02*	33.14	371.42*	77.50
SYP	1.02	0.25	5.22*	2.10	229.40*	22.54	143.94*	17.41	16.48*	4.47
TH	1.00	0.00	9.52	14.86	1218.08*	186.74	217.79*	45.71	348.31*	150.50
Endoderm markers										
AFP	1.00	0.00	2.10	0.00	1.44	0.00	1.54	0.00	1.37	0.00
FN1	1.00	0.06	1.41	0.10	0.02*	0.00	1.96	0.19	0.00*	0.00
FOXA2	1.00	0.00	150.00*	55.92	1.44	0.00	1.54	0.00	1.37	0.00
GATA4	1.00	0.00	11.93	19.67	7.22	11.56	9.14	12.35	1.37	0.00
GATA6	1.00	0.09	0.37*	0.17	0.00*	0.00	0.44*	0.04	0.02*	0.01
GCG	1.00	0.00	7.96	11.74	1.44	0.00	33.59*	22.17	1.37	0.00
IAPP	1.00	0.00	2.10	0.00	1.44	0.00	1.54	0.00	1.37	0.00
INS	1.00	0.00	2.10	0.00	1.44	0.00	12.67	22.26	1.37	0.00
IPF1	1.00	0.00	2.10	0.00	1.44	0.00	1.54	0.00	1.37	0.00
LAMA1	1.00	0.11	4.42*	0.86	78.49*	6.82	43.99*	2.79	46.49*	16.59
LAMB1	1.02	0.26	12.51*	2.40	0.29*	0.09	2.27*	0.77	3.89*	1.12
LAMC1	1.00	0.10	2.82*	0.10	1.54	0.33	3.01*	0.94	1.31	0.30
NODAL	1.00	0.00	12.16	11.62	16.27	11.25	1.54	0.00	1.37	0.00
PAX4	1.00	0.00	6.77	9.35	1.44	0.00	1.54	0.00	1.37	0.00
PTF1A	1.00	0.00	2.10	0.00	1.44	0.00	1.54	0.00	1.37	0.00
SERPINA1	1.03	0.30	0.79	0.53	0.24	0.00	1.52	1.17	0.99	0.68
SOX17	1.00	0.00	2.10	0.00	1.44	0.00	1.35	5.63	1.37	0.00
SST	1.25	1.00	52.58*	10.67	0.55	0.36	48.97*	8.70	0.92	0.42
TAT	1.00	0.00	2.10	0.00	255.86*	84.52	106.04*	45.87	1.37	0.00
Mesoderm markers										
ACTC	1.04	0.35	0.01*	0.00	0.02*	0.01	0.05*	0.01	0.01*	0.01
CD34	1.67	1.69	501.85*	61.88	45.17*	27.01	113.96*	39.39	13203.40*	5385.80
CDH5	1.00	0.00	4.12	4.06	16.69	8.07	32.41*	20.31	13447.65*	3220.80
COL1A1	1.01	0.12	2.28*	0.41	0.00*	0.00	0.50*	0.05	0.02*	0.00
COL2A1	3.56	6.27	103.52*	37.78	1813.86*	236.76	873.19*	259.80	3815.72*	839.02
DES	1.00	0.07	1.94	0.33	1.09	0.33	0.87	0.07	0.22*	0.08
FLT1	1.01	0.15	0.68	0.29	0.00	0.00	0.46*	0.05	0.00*	0.00
HBB	3.08	4.01	0.39	0.00	0.27	0.00	0.29	0.00	0.26	0.00
HBZ	1.14	0.63	3.53	1.32	0.25	0.22	0.61	0.63	2.88	1.20
HLXB9	1.00	0.00	2.10	0.00	59.80*	16.35	24.94	3.14	35.12	40.50
MYF5	1.77	1.87	0.69	0.00	0.47	0.00	0.51	0.00	0.45	0.00
MYOD1	1.71	2.27	1.22	0.00	0.83	0.00	0.89	0.00	0.80	0.00
NPPA	1.00	0.00	2.10	0.00	96.60*	76.23	18.97	26.98	32.37	10.96
PECAM1	1.00	0.00	1041.24*	150.95	31.30*	24.22	964.70*	200.82	7305.03*	1127.69
RUNX2	1.01	0.12	1.76	0.37	0.09*	0.02	0.78	0.23	1.18	0.27
T	1.00	0.00	2.10	0.00	1.44	0.00	1.54	0.00	1.37	0.00
WT1	2.09	3.13	1.11	0.00	0.76	0.00	2.72	3.82	4.24	4.21

For Relative Expression calculations, each sample was normalized to the average Ct of the 6 housekeeping genes (ACTB, 18S, CTNNB1, EEF1A1, GAPD, RAF1), and calibrated to the Untreated HFF (Passage 8) control.

Relative Expression values with asterisk (*) indicate values with significant up or down-regulation (>2-fold or <0.5-fold). For these samples, for Ct values <35 is considered that the expression of the gene is adequate for quantification.

For the Relative Expression values that are >2-fold or <0.5-fold but without asterisk, the values could have significant error due to the low expression of the gene (Ct > 35), and thus the up or down-regulation could be merely a result of the high standard deviation of the high Ct values of the genes, or fluctuations in the housekeeping genes.

As for the Relative Expression values that are between 0.5-fold and 2-fold, it indicates no significant change in the expression of the gene for these samples.

TABLE 8c

Results for relative expression of Embryonic Stem Cell, Ectoderm, Endoderm/mesoderm, and neuronal markers in untransfected and transfected HFF with Msi1/Ngn2/MBD2 calibrated to untreated HFF (passage 8).

Gene	Untreated HFF (Passage 8) Rel. Exp.	Std. Dev.	Untransfected HFF (Day 9) Rel. Exp.	Std. Dev.	hNPC neurospheres (Passage 4) Rel. Exp.	Std. Dev.	MSI1/NGN2/MBD2-transfected HFF (Day 9) (NSLC, Passage 1) Rel. Exp.	Std. Dev.	Neural stem-like cells, NSLC (Passage 5) Rel. Exp.	Std. Dev.
Embryonic Stem Cell Markers										
OCT4*	1.04	0.38	7.27	0.81	6.26	0.05	6.63	0.51	3.15	0.58
OCT4 (5'UTR)	1.04	0.41	0.08	0.00	2.07	0.11	1.82	0.53	0.55	0.59
NANOG (5'UTR)	1.02	0.32	19.29	2.23	11.27	0.89	16.73	6.86	9.94	6.32
FBXO15*	1.05	0.46	2.58	0.45	3.57	0.23	5.89	1.22	1.13	0.39
ALPL*	1.03	0.33	0.57	0.73	652.20	46.60	194.23	10.82	13.04	4.04
SALL4*	1.02	0.25	9.20	1.35	9.76	0.62	15.84	0.92	2.35	0.55
NR0B1 (DAX1)*	1.01	0.19	18.62	4.70	2.64	0.11	11.59	3.17	0.06	0.00
Ectoderm Markers										
ZIC1*	1.01	0.24	2.01	0.25	1889.80	93.48	1158.21	80.43	156.40	12.64
SOX1*	1.00	0.01	2.05	0.06	1776.83	128.63	1052.75	243.07	47.98	2.12
CDH1 (E-cadherin)*	1.00	0.01	2.05	0.06	264.59	6.22	59.14	7.57	18.20	3.73
p63	1.00	0.01	68.37	72.49	18.01	5.33	39.72	12.76	37.83	6.76
MSX1	1.00	0.05	4.19	0.56	0.10	0.01	1.53	0.35	0.09	0.00
NOTCH1*	1.00	0.07	1.26	0.08	7.38	1.20	4.51	0.54	4.75	0.26
SOX2*	1.00	0.01	2.50	0.57	340909.59	5659.15	194495.82	17929.15	219269.76	31399.68
SOX2 (3'UTR)*	1.00	0.01	7.74	8.11	864191.09	60204.44	452684.80	26457.70	618245.01	7107.48
Mesoderm/Endoderm Markers										
CXCR4*	1.05	0.46	12.45	5.64	5048.23	172.14	2763.82	30.29	3773.11	78.89
Neuronal markers										
MAP2*	1.01	0.17	2.98	0.20	155.33	9.08	88.82	6.48	27.38	0.13
TUBB3*	1.00	0.04	0.38	0.02	1.15	0.05	0.89	0.05	0.98	0.09
ASCL1 (MASH1)*	1.29	1.16	11.19	0.22	42618.46	68.52	23554.16	1588.45	31358.79	2301.26
NGN2*	1.00	0.01	2.05	0.06	19.45	6.64	247883.48	16409.80	968.11	191.73
NGN2 (3'UTR)*	1.83	2.17	1.17	0.76	13.39	5.10	8.45	1.75	539.02	59.72
MSI1*	1.00	0.01	263.87	70.10	100376.36	81.45	479098.05	2281.62	116105.29	2745.03
MSI1 (3'UTR)*	1.01	0.20	13.61	2.00	3601.96	345.79	2163.87	59.84	3698.14	160.78
ACHE*	1.00	0.00	2.00	0.26	25.00	3.71	12.84	0.84	21.30	0.30
Glia markers										
CNP*	1.01	0.18	1.43	0.10	3.48	0.58	2.69	0.12	1.93	0.07
SOX9*	1.00	0.04	3.54	0.06	88.25	9.71	41.11	2.70	26.96	0.53

Genes with asterisk (*) indicate that the Ct values of the test samples are within the quantifiable range (Ct < 35), suggesting the expression of the gene in the test sample is adequate for quantification.

For genes without asterisk, the values may be inaccurate due to the low expression of the gene (Ct > 35) and thus the up or down-regulation is merely a result of the high standard deviation of the high Ct values of the genes, or fluctuation of the housekeeping genes; the trend for these samples may be correct, but the absolute relative expression values may not. Expression of NGN3 and LIN28 were also tested but these two genes were not expressed in any of the test samples (data not shown).

RT-PCR revealed a significant increase of ectoderm and neuronal markers.

Note that custom primers (5'UTR) for detecting endogenous gene expression are generally not as sensitive and/or effective as standard primers (from the supplier's (Origene) catalog) that dtect overall gene expression (both endogenous and exogenous) of a particular gene.

In another part of the experiment, another batch of cells that were transfected with Msi1/Ngn2+pCMV6-XL5-MBD2 were plated on Poly-Ornithine (30 min at RT) and Laminin (1 h at RT) coated plates in CDM II medium in 5 different wells. On day 1, medium in two of the wells was switched to the same medium as in condition 1a (Table 7) until day 12. Medium was changed daily until day 12, at which point it was switched to either NS-A Differentiation Medium (StemCell Technologies) or NbActive4 (BrainBits™) medium that were both supplemented with BDNF (20 ng/ml), NT-3 (20 ng/ml), NGF (20 ng/ml), Retinoic acid (5 μM), Noggin (20 ng/ml) and Forskolin (10 μM). These cells showed a typical neural stem-like cell morphology by day 7, and proliferated until day 12. During the exposure to either of the two differentiation media, these NSLC changed to a more neuronal and glial phenotype as shown in the bright field pictures, but only expressed GFAP by Day 17.

For the other three wells, on day 1 medium was switched to either NS-A Differentiation Medium (StemCell Technologies), NbActive4 (BrainBits), or CDM II medium; these first two were supplemented with the same cytokines as previously described but with the addition of Fgf-2 (20 ng/ml). On day 12, Fgf-2 was removed from the first two differentiation media while cells in the CDM H medium were switched to the NS-A Differentiation Medium (StemCell Technologies) supplemented with cytokines without Fgf-2. Between day 12 and day 17, media was changed every two to three days. During the first 12 days of culture, cells in all 3 media developed into a mix of more spindle shaped cells compared to untransfected fibroblasts and some into cells with a NSLC morphology; upon removal of Fgf-2 cell morphology turned into a very pronounced neuronal shape as well as glial cells with a network established between cells as shown in the bright field pictures that expressed GFAP and βIII-tubulin by Day 17.

An additional study was designed to assess the effect of Msi1, Ngn2 and MBD2 on their endogenous proteins levels in reprogrammed cells. Cells were transfected with the MSI1/NGN2 vector and MBD2 as previously described and cultured in proliferation condition at 37° C., 5% CO_2 and 5% O_2. Samples were collected at various time points from Day 2-10 and analyzed by RT-PCR to investigate the expression of endogenous genes and the expression of neural stem cell and neuronal genes at different time points. RT-PCR revealed a gradual loss of total Msi1, Ngn2 and MBD2 gene expression starting from Day 2 to Day 10, with the increase in MBD2 expression relative to control having been almost completely lost by Day 5. This decrease was associated with a significant activation of endogenous Msi1 and Ngn2 on Day 5, with another jump in endogenous gene expression at Day 9 (Table 9). A significant increase in Sox2 expression was detected at Day 4, and the expression of this ectoderm/neural stem cell/neuronal gene continued to increase with each subsequent timepoint (Table 10). GFAP (a neural stem cell and astrocyte marker) was slightly elevated already from Day 2 onwards, but significantly increased on Day 5 with a large jump in gene expression at Day 7 analysis timepoint and stayed at this expression level for the rest of the study period. Expression of the neural stem cell marker Nestin also started to slowly increase from Day 5 onwards. Expression of the neuronal genes βIII-tubulin (TUBB3) and Map2b were slightly elevated already from Day 2 onwards, but significantly increased on Day 5 onwards. Expression of a marker for acetylcholine receptors (found in neurons), acetylcholine esterase (ACHE), was also slightly elevated from Day 2 onwards, but did not significantly increased until Day 7 onwards. It should be noted that among the neural stem cell markers that were analyzed, the relative expression of Sox2 was highly and early expressed which could then be directly or indirectly interact with the exogenous Msi1Ngn2 and/or other genes in the activation of Nestin, GFAP, and endogenous Msi1 and Ngn2 and other genes that promote the reprogramming and cell fate change, as well as the activation of neuronal genes like βIII-tubulin (TUBB3), Map2b, and ACHE.

TABLE 9

RT-PCR analysis of exogenous and endogenous relative expression of Msi1, Ngn2 and MBD2 from Day 2-10 after transfection of fibroblast cells with pCMV-Msi1-Ngn2(Msi1/Ngn2) and MBD2 and cultured for 10 days in proliferation medium. Cells were collected at different time point to analyse endogenous gene expression.

	MSI1		Endogenous MSI1		NGN2		Endogenous NGN2		MBD2		Endogenous MBD2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1 Day12 Untransfected HFF	1.01	0.18	1.04	0.38	1.01	0.15	1.01	0.15	1.01	0.21	1.00	0.14
#2 Day12 HFF Msi1/Ngn2 + MBD2	1102.17	91.80	620.56	19.49	2208.36	375.09	51.09	14.69	1.09	0.00	0.83	0.06
#3 Day18 HFF Msi1/Ngn2 + MBD2	1470.36	164.35	950.07	152.50	71.57	52.59	122.66	39.63	1.21	0.02	0.73	0.08
#4 Untransfected Keratinocytes	1.49	N/A	1.01	N/A	1.00	N/A	1.00	N/A	1.02	N/A	1.00	N/A
#5 Day 12 Keratinocytes Msi1/Ngn2 + MBD2	4142.78	872.87	364.20	60.90	4656.42	232.63	102.01	3.18	0.40	0.14	0.74	0.30
#6 Day 18 Keratinocytes Msi1/Ngn2 + MBD2	4830.20	291.17	486.38	19.59	50.01	6.99	43.08	13.78	0.40	0.01	0.67	0.01
#7 Untransfected CD34+	1.01	0.19	1.00	0.01	1.01	0.16	1.17	0.87	1.00	0.02	1.00	0.07
#8 Day 18 CD34+ Msi1/Ngn2 + MBD2	3969.52	286.36	147.99	7.08	2.03	0.55	3.72	1.23	0.43	0.06	0.90	0.18
hNPC (14 Oct. 2009, EXP0067)	7574.57	234.74	1141.14	49.15	8.18	5.64	6.27	5.19	0.58	0.00	2.35	0.03

TABLE 10

RT-PCR analysis of relative expression of Nestin, Map2b, TUBB3, ACHE, GFAP, and Sox2 from Day 2-10 after transfection of fibroblast cells with pCMV-Msi1-Ngn2 (Msi1/Ngn2) and MBD2 and cultured for 10 days in proliferation medium. Cells were collected at different time point to analyse endogenous gene expression.

	NES		MAP2		TUBB3		ACHE		GFAP		SOX2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1 Untransfected Day 2	1.00	0.04	1.00	0.01	1.00	0.03	1.00	0.08	1.01	0.23	1.17	0.87
#2 Msi1/Ngn2 + MBD2/+Noggin Day 2	0.88	0.01	8.59	0.18	1.38	0.03	5.71	1.06	4.56	0.08	1.26	0.82
#3 Untransfected Day 3	1.38	0.07	0.66	0.03	0.40	0.02	1.36	0.06	1.95	0.38	2.34	2.29
#4 Msi1-Ngn2 + MBD2/+Noggin Day 3	1.39	0.08	4.31	0.24	0.79	0.09	6.03	0.60	4.66	0.02	0.96	0.10
#5 Untransfected Day 4	2.43	0.23	1.78	0.11	0.44	0.01	2.70	0.02	3.76	0.86	0.93	0.01
#6 Msi1/Ngn2 + MBD2/+Noggin Day 4	1.91	0.06	2.81	0.20	0.64	0.02	6.76	0.64	8.67	1.06	5.37	6.06
#7 Untransfected Day 5	1.40	0.05	1.13	0.04	0.41	0.03	1.17	0.37	5.44	0.02	15.03	8.77
#8 Msi1-Ngn2 + MBD2/+Noggin Day 5	4.31	0.08	71.60	6.43	1.34	0.01	7.60	0.18	42.28	2.94	66377.25	4089.77
#9 Untransfected Day 7	2.24	0.00	4.02	0.15	1.22	0.05	1.10	0.48	7.61	1.24	1.34	0.02
#10 Msi1/Ngn2 + MBD2/+Noggin Day 7	3.07	0.11	48.10	2.85	2.70	0.05	13.11	1.30	3271.10	149.81	44255.59	2004.08

TABLE 10-continued

RT-PCR analysis of relative expression of Nestin, Map2b, TUBB3, ACHE, GFAP, and Sox2 from Day 2-10 after transfection of fibroblast cells with pCMV-Msi1-Ngn2 (Msi1/Ngn2) and MBD2 and cultured for 10 days in proliferation medium. Cells were collected at different time point to analyse endogenous gene expression.

	NES		MAP2		TUBB3		ACHE		GFAP		SOX2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#11 Untransfected Day 9	4.37	0.23	14.55	0.96	1.75	0.14	3.35	0.36	15.95	0.23	429.09	119.98
#12 Msi1-Ngn2 + MBD2/+Noggin Day 9	7.97	0.16	123.55	3.27	2.79	0.12	16.59	0.03	3152.25	3.31	114149.70	3372.20
#13 Untransfected Day 10	3.48	0.44	10.03	0.37	1.63	0.21	3.20	0.81	5.64	1.92	14.66	5.03
#14 Msi1/Ngn2 + MBD2/+Noggin Day 10	7.48	0.22	100.25	6.66	2.87	0.03	17.49	1.35	3374.03	22.47	101105.49	3996.44

Example IV

Comparison of the Nucleofector™® II Device and the Nucleofector™® 96-Well Shuttle® Device in the Reprogramming of HFF into NSLC in Adherent and Floating Conditions.

HFF cells were cultured as described in Example I, and transfected using the Nucleofector™® II Device (Lonza) as previously described in Example II or the Nucleofector™® 96-well Shuttle® Device (Lonza). The HFFs were harvested with TrypLE™ (Gibco), and 1×10^6 cells/transfection with the Nucleofector™® II Device for 10 min at 90 g and 6×10^5 cells/transfection with the Nucleofector™® 96-well Shuttle® Device for 5 min at 80×g. After centrifugation, the cell pellet was gently resuspended in either 100 µl of Basic Nucleofector™ Solution for the Nucleofector™® H or 20 µl of SE Solution (Cell line kit SE, Lonza) for the Nucleofector™® 96-well Shuttle®. For the Nucleofector™® II Device, each 100 µl of cell suspension was combined with 2 different mixes of plasmid DNA (sample 1 was mixed with 2 µg of pCMV6-XL5-Msi1 and 2 µg pCMV6-XL5-MBD2, and sample 2 with 2 µg of Msi1/Ngn2 and 2 µg pCMV6-XL5-MBD2). Each cell suspension was transferred into an Amaxa certified cuvette and transfected with the appropriate program (U-023). Right after transfection, 900 µl of warm CDM1 medium was added to each cuvette and the sample was transferred into a culture plate coated with Laminin (Stemgent, 10 µg/ml) at a cell density of 1×10^5 to 1.5×10^5 cells per cm^2 or into non-cell culture treated Petri dishes for neurosphere formation. The cells were incubated at 37° C., 5% CO_2 overnight. However for the Nucleofector™® 96-well Shuttle® Device, the steps described before were similar with the following exceptions: the cell suspension was mixed with 0.6 µg of each DNA of the same 2 DNA mixes, the cell suspension was transferred to a well of a 96-well Nucleoplate™ (Lonza) and transfected with the program FF-130™. After transfection, 80 µl of warm CDM1 medium was added to each well and the samples were left for 10 min in the incubator prior to being transferred into a laminin coated plate or non-cell culture treated Petri dishes at the same cell density as previously mentioned. For both devices, these steps were repeated for each sample that was transfected. Prior to transfection cells were cultured in CDM1 as described in Example I. After 24 hours, the medium was switched to a mix of 75% CDM medium and 25% Proliferation Medium which was supplemented with EGF (20 ng/ml), FGF-2 (20 ng/ml), Noggin (20 ng/ml) and Cytochalasin B (10 µg/ml) and the cells were incubated at 37° C., 5% CO_2 and 5% O_2. The medium was changed daily with an increased proportion of Neural proliferation medium up to 100% by Day 4 and a decreased proportion of Cytochalasin B that was completely omitted by Day 5. Forskolin (10 µM) was added to the medium from Day 4 onwards. The cells in floating conditions were pelleted by centrifugation and their medium changed daily as described for the adherent condition. Cells were collected at Day 3, 7, and 12 for immunohistochemistry analysis.

Fluorescence images were taken with a Cellomics™ ArrayScan HCS Reader™ microscopy system to determine an estimate of the percentage of cells positive for Sox2, a neural stem cell marker. This analysis revealed that in untransfected controls and at 3 days after transfection, no nuclear Sox2 staining was detectable. However, at Day 7 and Day 12 the percentage of Sox2 positive cells increased progressively under all transfection conditions except the pCMV6-XL5-Musashi and pCMV6-XL5-MBD2 Nucleofector™® II condition. The highest percentage at Day 12 was obtained with Msi1/Ngn2 and pCMV6-XL5-MBD2 transfected with the Nucleofector™® 96-well Shuttle® Device (~80%). The same combination transfected with the Nucleofector™® II yielded only ~35% positive cells. The pCMV6-XL5-Musashi and pCMV6-XL5-MBD2 with the Shuttle® produced ~20% positive cells, while generally none were observed with the Nucleofector™® II. The percentage of positive cells varied strongly between wells. The staining indicated that the cell population was not homogenous, since fields of densely arranged Sox2 positive cells and complete fields with only negative cells could be found in all cases. In general the Shuttle® was initially more toxic to cells than the Nucleofector™® II, however at least in the case of Msi1/Ngn2 and pCMV6-XL5-MBD2 shuttle, the Sox2 positive population rapidly expanded from Day 7 to Day 12 to have twice as many Sox2 positive cells as compared to the Nucleofector™® II. The cells in floating conditions did not form spheres during the 12 day experiment in any of the conditions, suggesting that the formation of neurospheres requires either the generation of neural stem-like cells in adherent conditions first or more time.

Table 11 shows the percentage of Sox2 positive cells with a typical neural stem cell morphology using both the Nucleofector™® II Device and the Nucleofector™® 96-well Shuttle® Device. The latter had the advantages of requiring a smaller starting material (less cells and less DNA required) and in addition gave rise to a higher number of Sox2 positive cells. Moreover a very small population of Sox2 positive cells was observed with the Shuttle® Device only upon transfection with only one neurogenic transcription factor (Msi) in the presence of the DNA demethylator MBD2.

TABLE 11

Percentage of positive cells for Sox2 after transfection of fibroblast cells with different expression vectors. After transfection the cells were cultured in proliferation medium (StemCell Technologies) supplemented by EGF (20 ng/ml, Peprotech) and FGF (20 ng/ml, Peprotech) for two weeks at 37° C./5% CO_2/5% O_2. The percentage of immunopositive cells was determined by Cellomics ™ and represented as mean ± SD (n = 3-5).

% Sox2 positive cells

		Day 3		Day 7		Day 12	
		Sox2	Total Cell count	Sox2	Total Cell count	Sox2	Total Cell count
Shuttle	MSI1/NGN2 + MBD2	1.34 ± 0.10	6430 ± 566	31 ± 20 ± 8.03	10683 ± 1112	78.17 ± 3.10	29341 ± 2527
	Msi + MBD2	1.08 ± 0.61	8253 ± 399	3.19 ± 3.57	8953 ± 672	19.05 ± 17.88	11082 ± 2999
Nucleofector ™	MSI1/NGN2 + MBD2	0.87 ± 0.30	21870 ± 4476	14.30 ± 1.83	37321 ± 6877	35.93 ± 7.10	33009 ± 1567
	Msi + MBD2	0.64 ± 0.07	46793 ± 8808	0.35 ± 0.16	34854 ± 2186	0.51 ± 0.25	32095 ± 3236

Example V

Neurosphere Formation Assay and Cell Differentiation Analysis

Based on previous studies showing that greater proportional reprogramming is achieved by transfecting two neurogenic genes, this study was designed to evaluate the number of reprogramming cells by using the vector Msi1/Ngn2, containing two neurogenic transcription factors (Msi1 and Ngn2) and the role of DNA demethylator or DNA methylation inhibitor (5-azacytidine) and histone deacetylation inhibitor (VPA) in the reprogramming process. HFFs were cultured and treated with cytochalasin B as described in Example III, and treated simultaneously with VPA (1 mM) and 5-Azacytidine (0.5 μM). After two days of treatment, cells were transfected by Nucleofection as described in Example II with the constructed vector Msi1/Ngn2. After preparing the cells, they were mixed with 2 μg of total DNA (Msi1/Ngn2) and cells that had not been treated with chemical inhibitors (VPA and 5-Aza) were co-transfect with MBD2 (2 μg), using the appropriate program (U023). The samples were transferred into a coated culture plate with Laminin (10 μg/ml, Sigma) and incubated in a humidified 37° C./5% CO_2/5% CO_2 incubator. The medium was changed to the proliferation basal media, Neural Proliferation Medium (NeuroCult™ proliferation Kit, StemCell Technologies), with the presence of Noggin (20 ng/ml, Peprotech), recombinant hFGF (20 ng/ml, Peprotech), and recombinant hEGF (20 ng/ml, Peprotech). Following 6 days of transfection, cells were harvested using Accutase™ (Millipore), centrifuged (300×g, 5 min, RT) and plated in uncoated cell culture dishes in NeuroCult™ NSC Proliferation medium to investigate the capacity to grow cells in suspension as neurospheres or on Laminin coated-plates for adherent culture. To prevent loss of floating spheres during media changes, cells were sedimented by centrifugation at 150×g for 3 min at room temperature (RT). The pellet was then resuspended in fresh medium and plated into new uncoated, low-bind cell culture dishes. Cultures were incubated at 37° C., 5% CO_2, 5% O_2 and were fed daily for at least two months.

To investigate whether a single cell from human neural precursor cells (hNPCs) and human NSLCs was able to generate a neurosphere (a standard test for proving that a cell is a neural stem cell), neurospheres were dissociated into single cells and these single cells were isolated and cultured in proliferation medium in suspension, and neurosphere formation was monitored by taking bright field images using light microscope (Nikon, 10×) and by Cellomics™. These cells started to proliferate and grew as spheres starting day 6 to day 10. Immunohistochemistry analysis of these spheres (Table 12) on Day 20, revealed immunopositive staining for the neural stem cells markers Sox2, Musashi, CD133, Nestin, and GFAP. Cells also stained positive for βIII-tubulin (a marker for neurons), O4 (a marker for oligodendrocytes), and GFAP (a marker for astrocytes), indicating the tri-potent differentiation potential of both sets of cells (NSLC and hNPC), and negative for NGFrec and NeuN (markers for differentiated neurons) indicating that the cells were not terminally differentiated.

TABLE 12

Percentage of positive cells for neural stem cells, and neuronal, astrocyte and oligodendrocyte lineage markers in neurospheres formed from single NSLCs and hNPCs cultured in proliferation medium (StemCell Technologies) supplemented by EGF (20 ng/ml, Peprotech) and FGF (20 ng/ml, Peprotech) for 20 days at 37° C./5% CO_2/5% O_2. The percentage of positive cells was determined by Cellomics ™ and represented as mean ± SD.

% of positive cells	NSLCs	hNPCs
Musashi	91.8 ± 6.8	88.6 ± 7.9
Nestin	78.6 ± 5.7	75.4 ± 12.0
GFAP	69.2 ± 7.4	78.6 ± 8.4
βIII-tubulin	85.6 ± 6.4	76.6 ± 8.4
P75	0	0
NeuN	0	0
O4	65.4 ± 6.6	71.4 ± 7.5
CD133	0	0

HFF cells were cultured as described in Example I, and transfected using the Nucleofector™ II device (Lonza) as described in Example II. Cells were co-transfected with pCMV6-XL5-Msi/pCMV6-XL4-Ngn2, pCMV-Msi1-Ngn2 with MBD2 or pre-treated with VPA/5aza. Cells were cultured in proliferation medium as suspension or adherent cultures. Gene expression analysis on 8 samples was performed as previously described in Example I with the customized Neuronal Markers 2 TLDA (Table 13) which profiled the expression of 48 genes (including three housekeeping genes: ACTIN, GAPDH and PPIA) in four major categories; 1) fibroblast specific genes; 2) neuronal lineage specific genes; 3) Neural stem cell marker specific genes; and 4) Genes for growth factors and their receptors.

TABLE 13

Neuronal Markers 2 TLDA Layout (Applied Biosystems)
Gene Symbols

	1	2	3	4	5	6	7	8	9	10	11	12
1	ACTB	PPIA	COL3A1	LOX	SI00A4	SYT1	SNAP25	NEUROD1	MBP	NKX2-2	GAPDH	OLIG2
2	VIM	SOX3	SOX9	PROM1	SOX1	SOX2	KLF4	POU5F1	STAT3	PIK3CG	GDNF	NGF
3	ACTB	PPIA	COL3A1	LOX	SI00A4	SYT1	SNAP25	NEUROD1	MBP	NKX2-2	GAPDH	OLIG2
4	VIM	SOX3	SOX9	PROM1	SOX1	SOX2	KLF4	POU5F1	STAT3	PIK3CG	GDNF	NGF
5	ACTB	PPIA	COL3A1	LOX	SI00A4	SYT1	SNAP25	NEUROD1	MBP	NKX2-2	GAPDH	OLIG2
6	VIM	SOX3	SOX9	PROM1	SOX1	SOX2	KLF4	POU5F1	STAT3	PIK3CG	GDNF	NGF
7	ACTB	PPIA	COL3A1	LOX	SI00A4	SYT1	SNAP25	NEUROD1	MBP	NKX2-2	GAPDH	OLIG2
8	VIM	SOX3	SOX9	PROM1	SOX1	SOX2	KLF4	POU5F1	STAT3	PIK3CG	GDNF	NGF
9	ACTB	PPIA	COL3A1	LOX	SI00A4	SYT1	SNAP25	NEUROD1	MBP	NKX2-2	GAPDH	OLIG2
10	VIM	SOX3	SOX9	PROM1	SOX1	SOX2	KLF4	POU5F1	STAT3	PIK3CG	GDNF	NGF
11	ACTB	PPIA	COL3A1	LOX	SI00A4	SYT1	SNAP25	NEUROD1	MBP	NKX2-2	GAPDH	OLIG2
12	VIM	SOX3	SOX9	PROM1	SOX1	SOX2	KLF4	POU5F1	STAT3	PIK3CG	GDNF	NGF
13	ACTB	PPIA	COL3A1	LOX	SI00A4	SYT1	SNAP25	NEUROD1	MBP	NKX2-2	GAPDH	OLIG2
14	VIM	SOX3	SOX9	PROM1	SOX1	SOX2	KLF4	POU5F1	STAT3	PIK3CG	GDNF	NGF
15	ACTB	PPIA	COL3A1	LOX	SI00A4	SYT1	SNAP25	NEUROD1	MBP	NKX2-2	GAPDH	OLIG2
16	VIM	SOX3	SOX9	PROM1	SOX1	SOX2	KLF4	POU5F1	STAT3	PIK3CG	GDNF	NGF

	13	14	15	16	17	18	19	20	21	22	23	24
1	ALDHIL1	DIO2	GFAP	NCAM1	FOXJ1	PDGFRA	MKI67	NES	CSPG4	DLX2	MSI1	CROCC
2	BDNF	CNTFZFP91-CNTF	GAP43	NRG1	NPY	CSF3	BMP4	TGFB1	VEGFA	NGFR	EGFR	KDR
3	ALDHIL1	DIO2	GFAP	NCAM1	FOXJ1	PDGFRA	MKI67	NES	CSPG4	DLX2	MSI1	CROCC
4	BDNF	CNTFZFP91-CNTF	GAP43	NRG1	NPY	CSF3	BMP4	TGFB1	VEGFA	NGFR	EGFR	KDR
5	ALDHIL1	DIO2	GFAP	NCAM1	FOXJ1	PDGFRA	MKI67	NES	CSPG4	DLX2	MSI1	CROCC
6	BDNF	CNTFZFP91-CNTF	GAP43	NRG1	NPY	CSF3	BMP4	TGFB1	VEGFA	NGFR	EGFR	KDR
7	ALDHIL1	DIO2	GFAP	NCAM1	FOXJ1	PDGFRA	MKI67	NES	CSPG4	DLX2	MSI1	CROCC
8	BDNF	CNTFZFP91-CNTF	GAP43	NRG1	NPY	CSF3	BMP4	TGFB1	VEGFA	NGFR	EGFR	KDR
9	ALDHIL1	DIO2	GFAP	NCAM1	FOXJ1	PDGFRA	MKI67	NES	CSPG4	DLX2	MSI1	CROCC
10	BDNF	CNTFZFP91-CNTF	GAP43	NRG1	NPY	CSF3	BMP4	TGFB1	VEGFA	NGFR	EGFR	KDR
11	ALDHIL1	DIO2	GFAP	NCAM1	FOXJ1	PDGFRA	MKI67	NES	CSPG4	DLX2	MSI1	CROCC
12	BDNF	CNTFZFP91-CNTF	GAP43	NRG1	NPY	CSF3	BMP4	TGFB1	VEGFA	NGFR	EGFR	KDR
13	ALDHIL1	DIO2	GFAP	NCAM1	FOXJ1	PDGFRA	MKI67	NES	CSPG4	DLX2	MSI1	CROCC
14	BDNF	CNTFZFP91-CNTF	GAP43	NRG1	NPY	CSF3	BMP4	TGFB1	VEGFA	NGFR	EGFR	KDR
15	ALDHIL1	DIO2	GFAP	NCAM1	FOXJ1	PDGFRA	MKI67	NES	CSPG4	DLX2	MSI1	CROCC
16	BDNF	CNTFZFP91-CNTF	GAP43	NRG1	NPY	CSF3	BMP4	TGFB1	VEGFA	NGFR	EGFR	KDR

Sample Information

Sample ID	Sample Name	TLDA Port
1	HFF Ctrl	1
2	ReNcell Undifferentiated Ctrl	2
3	Msi1-Ngn2/MBD2	3
4	Msi1-Ngn2/MBD2	4
5	Msi1-Ngn2/VPA + AZA	5
8	Msi1-Ngn2	6
7	Msi1-Ngn2/MBD2, neurospheres	7
8	Msi1-Ngn2/MBD2, neurospheres	8

As shown in Table 14, fibroblast-specific genes (Col3A1, Lox, S100A4) were down-regulated in reprogrammed cells, indicating the loss of fibroblast-specific genes following transfection (note that not all cells got transfected and reprogrammed, so the presence of fibroblast-specific gene expression in the cultures is mostly from the un-programmed fibroblasts left in the culture). The expression of these genes is observed to increase when HFFs were transfected in the absence of DNA demethylator or the DNA methylation inhibitor, indicating that down-regulation of differentiated markers of fibroblast cells requires DNA demethylation. The expression of ectoderm genes such as Msi1, Sox2, and Nestin was remarkably increased following transfection in conjunction with DNA demethylation. The expression of neuronal markers, such as synaptogamin1 (a synaptic vesicle protein) and NeuroD1 was up-regulated in transfected cells with Msi1/Ngn2/MBD2, and slightly increased in transfected cells with Msi1/Ngn2/VPA and 5-AZA. The selected three markers of oligodendrocytes were detected in the transfected cells with a strong increase of Olig2. Two markers for astrocytes, GFAP and ALDH1L1, were enhanced following transfection. The results support the idea that neurospheres are composed of heterogeneous progenitor subtypes.

Among the neurotrophic factors, expression of CNTF was slightly increased in the reprogrammed cells. The expression of GAP-43 and neuropeptide Y (NPY) were the most annotated genes. GAP-43 has long been acknowledged to play a pivotal role in axonal plasticity and is used as a marker of regenerating neurite outgrowth and synaptogenesis, both in embryonic development and in neuronal regeneration in injured brain and spinal cord. Expression of receptors for growth and neurotrophic factors was increased, such as neurotrophic receptor tyrosine kinase expression.

TABLE 14

Gene array analysis was performed after one month of transfection of human fibroblast cells with Msi1/Ngn2, in the presence MBD2 or VPA and 5-Aza. Cells were cultured on coated culture plates as adherent cells or on untreated culture plates as neurospheres in proliferation medium (StemCell Technologies) supplemented with EGF (20 ng/ml) and FGF (20 ng/ml). Untransfected cells were considered as negative control and ReNcell (Millipore) as positive control.

		Relative expression to #1 HFF Ctrl						
Symbol	Common name and description	#2 ReNcell Undiff	#3 Msi1-Ngn2/ MBD2	#4 Msi1-Ngn2/ MBD2	#5 Msi1-Ngn2/ VPA + AZA	#6 Msi1-Ngn2	#7 Msi1-Ngn2/ MBD2, neurospheres	#8 Msi1-Ngn2/ VPA + AZA, neurospheres
Fibroblast/ECM component								
COL3A1	Collagen, type III, alpha 1, fibroblast marker	0.00	0.03	0.02	0.02	11.92	0.00	0.00
LOX	Lysyl oxidase, ECM component	0.01	0.03	0.01	0.01	2.38	0.00	0.00
FSP1	Fibroblast transcription site-1, enzyme for ECM remodeling	0.04	0.04	0.06	0.05	3.22	0.05	0.05
Neuron markers								
SYT1	Synaptotagmin1, a synaptic vesicle protein in neurons	106.49	108.40	78.66	26.72	22.42	37.61	16.80
SNAP25	SNAP25, mature neuron marker	4.72	6.10	7.89	3.11	3.19	6.47	4.00
NEUROD1*	Neurogenic differentiation 1, neuron marker	2.32	93.35	100.84	2.02	3.11	271.11	10.23
Oligodendrocyte markers								
MBP*	Myelin Basic Protein, mature oligodendrocyte marker	2.32	48.53	18.11	6.94	667.56	16.67	1.67
NKX2-2*	NK2 homeobox 2, remyelination	2.32	75.31	54.65	1.66	3.11	1.67	1.74
OLIG2*	Oligodendrocyte lineage transcription factor 2, oligodendrocyte progenitor	2856.4	15594	67369	38733	3.11	92420	101733
Astrocyte markers								
ALDH1L1*	Aldehyde dehydrogenase 1 family member L1, astrocyte	6.20	3.77	4.65	1.66	0.02	5.87	9.59
DIO2*	Deiodinase iodothyronine type II, astrocyte marker	23.20	0.00	0.00	0.00	0.51	0.00	0.00
GFAP	Glial fibrillary acidic protein, astrocyte marker	3342.1	6899.0	6291.0	4800.9	1.27	3118.7	3222.0
NSCS markers								
NCAM1	NCAM1, neuroblast marker	23.21	43.90	24.45	12.72	1.13	31.93	36.70
PDGFRA	Plate-derived growth factor receptor alpha, oligodendrocyte progenitor cells	0.05	0.01	0.01	0.00	4.42	0.00	0.01
NES	Nestin, neural progenitor	5.76	19.84	19.56	3.46	4.23	16.57	8.36
MSI1*, **	Musashi I, neuroblast marker	5120.3	5985.2	5262.7	5645.1	204.34	3179.6	4113.6
SOX1*	Sox1, neural progenitor	679.21	223.59	373.14	361.67	3.11	287.82	323.23
SOX2*	Sox2, NSCs	1924084	2265299	1889166	1014816	3.11	1313765	1103212
Neurotrophic/ Growth Factor								
GDNF*	Glial cell derived neurotrophic factor	0.01	0.02	0.02	0.00	1.69	0.00	0.00
NGF*	Nerve growth factor	0.00	0.00	0.00	0.00	1.48	0.00	0.00
BDNF	Brain derived neurotrophic factor	0.03	0.09	0.09	0.05	0.82	0.02	0.01
CNTF*	Ciliary neurotrophic factor	9.25	4.32	3.11	2.90	64.05	2.31	3.39
GAP43	Growth associated protein 43, neural regeneration	917.52	3506.5	1530.8	452.75	584.00	746.25	578.52
NRG1*	Neuregulin 1, neural regeneration	0.01	0.00	0.00	0.00	0.40	0.00	0.00
NPY*	Neuropeptide Y, interneuron	2.32	675.69	465.04	153.54	3.11	1244.0	130.38
CSF3*	Colony stimulating factor 3, neural regeneration	0.50	0.03	0.02	0.58	18.62	0.02	0.02
BMP4	Bone morphogenetic protein 4, remyelination marker	0.83	0.26	0.74	0.45	11.03	0.09	0.07
TGFB1	Transforming growth factor, beta 1	0.85	2.39	0.92	0.83	0.65	0.45	0.58
Angiogenesis								
VEGFA	Vascular endothelial growth factor	2.77	14.93	15.01	2.67	3.82	2.80	3.21

TABLE 14-continued

Gene array analysis was performed after one month of transfection of human fibroblast cells with Msi1/Ngn2, in the presence MBD2 or VPA and 5-Aza. Cells were cultured on coated culture plates as adherent cells or on untreated culture plates as neurospheres in proliferation medium (StemCell Technologies) supplemented with EGF (20 ng/ml) and FGF (20 ng/ml). Untransfected cells were considered as negative control and ReNcell (Millipore) as positive control.

		Relative expression to #1 HFF Ctrl						
Symbol	Common name and description	#2 ReNcell Undiff	#3 Msi1-Ngn2/ MBD2	#4 Msi1-Ngn2/ MBD2	#5 Msi1-Ngn2/ VPA + AZA	#6 Msi1-Ngn2	#7 Msi1-Ngn2/ MBD2, neurospheres	#8 Msi1-Ngn2/ VPA + AZA, neurospheres
Neurotrophin/ Growth Factor								
Receptors								
NGFR/P75	NGFR, neurotrophin receptor	5.35	3.29	5.78	9.10	7.53	7.26	17.51
EGFR	Epidermal growth factor receptor	0.89	0.77	0.86	0.79	1.63	1.44	1.25
KDR*	Kinase insert domain receptor, growth factor receptor	210.87	259.42	263.45	51.85	0.07	11.23	17.50

Further analysis and quantification of the adherent population of NSLCs showed that cells were positively stained for Sox2 (93.43±1.9%), nestin (60.76±5.7%), and GABA (37.48±4.9), while these markers were undetectable in untransfected cells (Table 15). Furthermore, these cells stained positive for p75NTR (31.15±1.6), βIII-tubulin (37.55±0.6%) and GFAP (16.47±0.9). However, untransfected HFFs only stained positive for HFF markers, such as fibronectin and fibroblast protein marker, while these markers were undetectable in reprogrammed cells, demonstrating that the reprogrammed cells lost markers of the original cells and adopted morphology and markers of neural stem cells and a neuronal lineage.

TABLE 15

The percentage of cells stained positive for neural stem cell markers and fibroblast markers in untransfected cells and transfected cells with pMsi1/Ngn2/MBD2. Transfected cells (NSLCs) possess a high percentage of neural stem markers but a very low percentage of fibroblast markers as compared to untransfected cells. The percentage of immunopositive cells was determined by Cellomics ™ and represented as mean ± SD (n = 5).

Marker protein	Transfected fibroblast cells (% of average positive cells ± stdv)	Untransfected fibroblast cells (% of average positive cells ± stdv)
Sox2	93.43 ± 1.9	1.90 ± 0.5
Nestin	60.76 ± 5.7	0.84 ± 0.2
p75NTR	31.15 ± 1.6	3.95 ± 1.7
NCAM	26.84 ± 3.8	0.87 ± 0.2
S100	41.80 ± 0.6	1.60 ± 0.3
GFAP	16.47 ± 0.9	3.84 ± 0.9
βIII-Tubulin	37.55 ± 0.6	1.90 ± 0.9
GABA	37.48 ± 4.9	2.54 ± 0.5
Fibronectin	1.05 ± 0.7	94.19 ± 0.9
Fibroblast marker protein	4.81 ± 1.0	50.30 ± 7.8

This study showed as well that NSLCs have the capacity to proliferate in culture and exhibit stable morphology, gene and protein expression that were maintained for the entire study period, which was for over five month in culture (Table 16).

TABLE 16

Doubling time of NSLCs over serial passages. NSLCs were maintained in proliferation conditions for 35 passages in a 37° C., 5% CO_2 and 5% O_2 incubator. The time required for the cell population to double (g) was calculated for each passage, and was defined as g = (ln2)/k, where k was the number of generations that occured per unit time (t) defined as, k = (ln N_f − ln N_0)/t, where N_f was the final cell number and N_0 the initial seeded cell number. The average generation time was 25.4 h over 35 passages.

Passage number	Time (h)	LN N_0	LN N_f	k (h^{-1})	g (h)
2	168	11.513	15.577	0.024	38.655
3	216	11.513	16.195	0.022	31.977
4	192	11.513	18.258	0.035	39.730
5	144	11.513	16.258	0.033	21.036
6	144	11.513	16.258	0.033	21.036
7	144	11.513	15.702	0.029	33.824
8	168	11.513	15.870	0.026	26.729
9	120	11.513	16.811	0.031	32.548
10	144	11.513	15.415	0.027	35.580
11	120	13.122	15.895	0.023	30
12	120	11.513	15.747	0.035	19.645
13	168	11.513	15.870	0.026	26.729
14	168	12.429	15.870	0.020	23.847
15	168	11.513	15.520	0.024	29.059
16	192	11.513	16.167	0.024	28.596
17	144	11.513	15.239	0.026	36.791
18	168	11.513	15.790	0.025	37.229
19	120	13.122	15.870	0.023	30.276
20	144	13.122	16.249	0.022	31.922
21	96	13.122	15.761	0.027	25.214
22	120	13.122	15.870	0.023	30.276
23	120	13.122	15.761	0.022	31.518
24	96	13.122	15.687	0.027	25.943
25	96	13.122	16.013	0.030	23.022
26	96	13.122	16.067	0.031	22.599
27	96	13.122	16.300	0.033	20.938
28	120	13.122	16.482	0.028	24.752
29	96	13.122	16.380	0.034	20.424
30	96	13.122	16.300	0.033	19.938
31	120	13.122	16.483	0.028	22.752
32	96	13.122	16.062	0.031	20.640
33	96	13.122	16.300	0.033	20.938
34	96	13.122	16.077	0.031	15.519
35	96	13.122	16.077	0.031	15.519

Gene Expression Microarray

Microarray expression analysis was performed to get a global overview to compare the gene expression profile of passage 7 NSLC to both HFF (the cells that the NSLC were created from) and hNPCs. NSLC (n=3), HFF (n=2), and hNPC (n=3) were resuspended in RNAlater™ (Qiagen) and shipped to Genotypics (India) where the samples were processed and the Gene Expression Microarray was performed.

In brief, Genotypics extracted RNA from the samples and performed Quality Control using an Agilent Bioanalyzer™. Labelling was done using Agilent's Quick Amp™ kit (cDNA synthesis and in vitro transcription), followed by Labelling QC. Hybridization was then performed using the 8×60K array, and scanning was done using high throughput Agilent scanner with SureScan™ technology. The Agilent Feature Extraction software was used for automated feature extraction, followed by Raw Data QC and Image QC. Advanced Data Analysis was then performed, including Pathway and Gene Ontology analysis using Agilent's GeneSpring GX™ v10.0 and Genotypic's Biointerpreter Software. The NSLC samples were compared to the HFF samples (Set 1) and hNPC samples (Set 2) The NSLC samples had a global gene expression pattern that was much closer to the hNPCs than the HFFs from which the NSLCs were created. Pearson correlation analysis revealed that NSLCs are closely related to hNPCs including in terms of neuronal lineage markers, regenerative genes and migration genes. These data confirm that NSLCs are similar, but not identical, to hNPCs.

Microarray analysis revealed an up-regulation of neural precursor genes in the NSLC samples as compared to the HFF samples. ACTL6A and PHF10, which both belong to the neural progenitors-specific chromatin remodelling complex (npbaf complex) and are required for the proliferation of neural progenitors, were up-regulated by 2.9-fold and 2.3 fold respectively. MSI2, which plays a role in the proliferation and maintenance of stem cells in the central nervous system, was up-regulated by 6-fold (Table X1). Glia genes were up-regulated in the NSLC samples as compared to the HFF samples. GFAP, is a neural stem cell- and astrocyte-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells, is highly up-regulated in the NSLC sample as compared to HFF (690-fold). OLIG1, which promotes formation and maturation of oligodendrocytes, especially within the brain, is also highly up-regulated in NSLC sample as compared to HFF (370-fold) (Table X2).

Table X3 lists a subset of regenerative genes that are up-regulated in the NSLC samples as compared to the HFF samples. SOX2, a gene critical for early embryogenesis and for embryonic stem cell pluripotency as well as neural stem cells, is highly up-regulated in the NSLC samples as compared to the HFF samples (5000-fold). CCND2, which is essential for the control of the cell cycle at the G1/S (start) transition, is also up-regulated in NSLC samples (70-fold as compared to TIFF samples). As shown in Table X4, numerous fibroblast genes were down-regulated in the NSLC samples as compared to the HFF samples. This shows that the NSLC lose the expression of numerous fibroblast genes as it gets reprogrammed from HFF to NSLC.

Table X5 show that neural precursor genes were also up-regulated in the NSLC samples as compared to the hNPC samples. BDNF, which promotes the survival and differentiation of selected neuronal populations of the peripheral and central nervous systems during development, is even more highly expressed in NSLC samples than in hNPC samples (34-fold up-regulation). Table X6 shows that a subset of Glia genes are also up-regulated in the NSLC samples as compared to the hNPC samples. GFAP, a neural stem cell- and astrocyte-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells, is more highly expressed in NSLC samples than hNPC samples (13-fold). PLP1, the major myelin protein of the central nervous system which plays an important role in the formation or maintenance of the multilamellar structure of myelin, is also more highly expressed in NSLC samples than in hNPC samples (20-fold).

Regenerative genes were also up-regulated in the NSLC samples as compared to the hNPC samples (Table X7). BMP2, a neural crest marker, but which induces growth especially of cartilage and bone formation and BMP4, which in turn induces cartilage and bone formation and acts in mesoderm induction, tooth development, limb formation and fracture repair, but also in neural stem cells, were both more highly expressed in NSLC samples than in hNPC samples (18-fold and 20-fold respectively). GAP43, which is a major component of the motile growth cones that form the tips of elongating axons was more highly expressed in NSLC samples than hNPC samples (4-fold). This suggests the regenerative potential of NSLC. HOXB4, a transcription factor that is involved in development and also in the expansion of neural stem cells as well as hematopoietic stem and progenitor cells in vivo and in vitro making it a potential candidate for therapeutic stem cell expansion, was also more highly expressed in NSLCs than in hNPCs. This data indicates that NSLCs are more 'stem-like' or have more 'stemness' than hNPCs.

TABLE X1

Up-regulated Neural Precursor genes (NSLC vs. HFF)

GeneSymbol	Accession Number	Fold change of NSLC compared to HFF[1]	p-value
ACTL6A	NM_178042	2.90	0.000
ADAM9	NM_001005845	2.64	0.004
AIFM1	NM_004208	2.45	0.000
BCAT1	NM_005504	3.23	0.000
BMP2	NM_001200	17.49	0.000
DLL1	NM_005618	40.32	0.000
EDNRB	NM_003991	933.03	0.000
ERBB4	NM_005235	53.22	0.006
GMNN	NM_015895	4.42	0.000
HES5	BC087840	102.33	0.000
KIF1B	NM_015074	9.45	0.002
LIMK1	NM_002314	2.44	0.002
MAPK8IP1	NM_005456	5.88	0.001
MCHR1	NM_005297	68.19	0.001
MEF2C	NM_002397	2.91	0.000
MSI2	NM_170721	6.76	0.000
NMB	NM_021077	3.65	0.000
NOS2A	NM_000625	279.45	0.000
NOTCH1	NM_017617	6.75	0.000
NPAS3	NM_022123	187.85	0.000
PHF10	NM_018288	2.28	0.001
PHLPP	NM_194449	8.84	0.000
SMAD1	NM_005900	4.74	0.000
SNTG1	AL161971	34.05	0.000
SP8	NM_198956	1392.67	0.000
STAU2	AK002152	3.35	0.000
STIL	NM_003035	4.94	0.003

[1]Fold change represents the up-regulation of the gene in the NSLC samples as compared to the HFF samples. (n = 2 for HFF samples, n = 3 for NSLC samples).

TABLE X2

Up-regulated Glia genes (NSLC vs. HFF)

GeneSymbol	Accession Number	Fold change of NSLC compared to HFF[1]	p-value
ASTN1	NM_004319	51.44	0.000
ATP1B2	NM_001678	186.64	0.000
B3GAT1	NM_018644	1784.49	0.000
BCL2	NM_000633	2.65	0.002
BMP7	NM_001719	41.35	0.000
CA14	NM_012113	43.44	0.000
CLCN2	NM_004366	4.18	0.000
CNDP1	NM_032649	4.39	0.010
CP	NM_000096	93.08	0.002

TABLE X2-continued

Up-regulated Glia genes (NSLC vs. HFF)			
GeneSymbol	Accession Number	Fold change of NSLC compared to HFF[1]	p-value
CXCR4	NM_001008540	4124.29	0.000
ERBB4	NM_005235	53.22	0.006
FABP7	NM_001446	18702.36	0.000
GAB1	NM_207123	2.44	0.001
GFAP	NM_002055	696.51	0.000
GJB2	NM_004004	13.89	0.001
ITGB8	NM_002214	8.48	0.005
KCNJ10	NM_002241	263.42	0.000
LMO3	NM_018640	194.32	0.000
MAP6D1	NM_024871	3.99	0.000
MAPT	NM_016835	2.38	0.001
NDE1	NM_017668	2.21	0.002
NEFL	NM_006158	10.30	0.001
NKX6-2	NM_177400	10.83	0.026
NOVA2	NM_002516	7.51	0.000
NTN1	NM_004822	5.29	0.015
NTRK3	NM_001012338	15.32	0.000
OLIG1	NM_138983	372.11	0.000
OLIG2	NM_005806	163.20	0.000
PARD6A	NM_016948	4.12	0.001
PASK	NM_015148	3.89	0.001
PAX6	NM_001604	28.53	0.001
PDCD11	ENST00000369797	2.23	0.001
PDE6B	NM_000283	5.55	0.001
PER1	NM_002616	2.43	0.001
PLP1	M54927	351.09	0.000
PTK2	NM_153831	4.22	0.000
QKI	NM_206855	8.75	0.003
S100B	NM_006272	456.00	0.000
SLC1A3	NM_004172	49.49	0.000
SORL1	NM_003105	27.61	0.000
SOX9	NM_000346	27.82	0.000
SPRY2	NM_005842	15.83	0.000
TARDBP	NM_007375	2.69	0.005
TSPAN12	NM_012338	259.78	0.000

TABLE X3

Up-regulated Regenerative genes (NSLC vs. HFF)			
GeneSymbol	Accession Number	Fold change of NSLC compared to HFF[1]	p-value
BMP2	NM_001200	17.49	0.000
CCND2	NM_001759	72.79	0.000
DLL1	NM_005618	40.32	0.000
EGR1	NM_001964	2.19	0.000
GAL	NM_015973	25.93	0.000
GAP43	NM_002045	1297.42	0.000
HOXB4	NM_024015	102.34	0.000
NFE2L2	AF323119	2.80	0.004
NOTCH1	NM_017617	6.75	0.000
PRPH	NM_006262	6.44	0.000
SEMA3A	NM_006080	3.03	0.004
SEMA6A	NM_020796	23.58	0.000
SOX2	NM_003106	5165.92	0.000

TABLE X4

Down-regulated Fibroblast genes (NSLC vs. HFF)			
GeneSymbol	Accession Number	Fold change of NSLC compared to HFF[1]	p-value
ACOT2	NM_006821	0.30	0.000
AEBP1	NM_001129	0.16	0.001
AGA	NM_000027	0.35	0.000
ANXA2	NM_001002857	0.26	0.029
AP4E1	NM_007347	0.30	0.008
APOE	NM_000041	0.08	0.000

TABLE X4-continued

Down-regulated Fibroblast genes (NSLC vs. HFF)			
GeneSymbol	Accession Number	Fold change of NSLC compared to HFF[1]	p-value
ARHGDIB	NM_001175	0.24	0.009
ASAH1	NM_004315	0.31	0.000
BDKRB1	NM_000710	0.00	0.001
BDKRB2	NM_000623	0.00	0.000
BDNF	NM_170735	0.12	0.000
BMP4	NM_001202	0.28	0.001
C3	NM_000064	0.25	0.001
C5orf13	NM_004772	0.18	0.000
CACNA1C	NM_000719	0.03	0.000
CASP4	NM_033306	0.00	0.000
CASP5	NM_004347	0.00	0.001
CCL2	NM_002982	0.20	0.000
CD36	NM_001001547	0.07	0.023
CDC42EP2	NM_006779	0.06	0.000
CDC42EP3	NM_006449	0.41	0.000
CDC42EP5	NM_145057	0.41	0.040
CDH11	NM_001797	0.00	0.000
CEMP1	AL833099	0.30	0.001
CFH	NM_001014975	0.01	0.010
CITED2	NM_006079	0.14	0.000
COL12A1	NM_004370	0.00	0.001
COL1A1	NM_000088	0.01	0.000
COL1A2	NM_000089	0.00	0.001
COL3A1	NM_000090	0.00	0.001
COL5A1	NM_000093	0.00	0.000
CPT1A	NM_001876	0.16	0.002
CROT	NM_021151	0.27	0.002
CTSA	NM_000308	0.10	0.000
CTSB	NM_147780	0.11	0.001
CXCL1	NM_001511	0.01	0.003
CXCL12	NM_000609	0.00	0.001
CYP27A1	NM_000784	0.28	0.011
CYR61	NM_001554	0.10	0.000
DCHS1	NM_003737	0.29	0.000
DMPK	NM_004409	0.36	0.000
DPT	NM_001937	0.05	0.006
EFEMP1	NM_004105	0.00	0.000
ELN	NM_000501	0.13	0.001
EMX2	NM_004098	0.00	0.001
EPS8	NM_004447	0.18	0.000
ETS1	NM_005238	0.15	0.003
FAH	NM_000137	0.17	0.000
FAM14A	NM_032036	0.22	0.001
FAP	NM_004460	0.00	0.000
FBLN2	NM_001004019	0.18	0.000
FBN1	NM_000138	0.01	0.002
FGF1	NM_000800	0.20	0.004
FGF13	NM_004114	0.04	0.006
FGF2	NM_002006	0.06	0.000
FGF5	NM_004464	0.01	0.003
FGF7	NM_002009	0.04	0.001
FGF9	NM_002010	0.01	0.000
FGFR1	NM_023110	0.34	0.026
FHL2	NM_201555	0.11	0.000
FN1	NM_212482	0.00	0.001
FSTL1	NM_007085	0.09	0.000
GADD45B	NM_015675	0.09	0.001
GALNT6	NM_007210	0.13	0.001
GAS6	NM_000820	0.02	0.000
GBA	NM_001005749	0.22	0.002
GBAP	NR_002188	0.19	0.000
GCH1	NM_000161	0.22	0.001
GGTA1	NR_003191	0.28	0.013
GIT2	NM_057169	0.37	0.003
GJA1	NM_000165	0.46	0.001
GLIS1	NM_147193	0.02	0.000
GM2A	AK127910	0.25	0.010
GNS	NM_002076	0.29	0.000
GPC3	NM_004484	0.22	0.038
GREM1	NM_013372	0.00	0.011
GSTM1	NM_146421	0.27	0.001
HAAO	NM_012205	0.43	0.001
HERPUD1	NM_014685	0.19	0.000
HEXA	NM_000520	0.24	0.000
HEXB	NM_000521	0.36	0.000

TABLE X4-continued

Down-regulated Fibroblast genes (NSLC vs. HFF)			
GeneSymbol	Accession Number	Fold change of NSLC compared to HFF[1]	p-value
HGF	NM_001010932	0.09	0.028
HGS	NM_004712	0.26	0.029
HIF1A	NM_181054	0.36	0.005
HLA-A	NM_002116	0.31	0.002
HLA-H	NR_001434	0.19	0.001
HOXB13	NM_006361	0.03	0.004
HR	NM_005144	0.18	0.002
HSPG2	NM_005529	0.19	0.004
IDUA	NM_000203	0.16	0.000
IGF1	NM_000618	0.10	0.004
IGFBP7	NM_001553	0.28	0.040
IKBKG	NM_003639	0.42	0.001
IRF1	NM_002198	0.28	0.002
ITGA1	NM_181501	0.00	0.001
ITGB3	NM_000212	0.05	0.000
KLF4	NM_004235	0.05	0.002
LEP	NM_000230	0.07	0.001
LEPRE1	NM_022356	0.24	0.000
LMNA	NM_005572	0.42	0.000
LOX	NM_002317	0.01	0.000
LOXL4	NM_032211	0.10	0.003
LRRC8C	NM_032270	0.15	0.013
MAGEL2	AJ243531	0.31	0.002
MAN2B1	NM_000528	0.45	0.006
MAP3K8	NM_005204	0.27	0.001
MEIS2	NM_170677	0.00	0.001
MKNK1	NM_003684	0.37	0.005
MMP1	NM_002421	0.00	0.000
MMP14	NM_004995	0.07	0.001
MMP2	NM_004530	0.04	0.000
MMP3	NM_002422	0.00	0.001
MOXD1	NM_015529	0.24	0.000
MRAS	NM_012219	0.15	0.001
MSX2	NM_002449	0.15	0.031
MTHFR	NM_005957	0.27	0.014
MYC	NM_002467	0.05	0.000
MYL6	NM_079423	0.33	0.001
MYL9	NM_181526	0.01	0.000
NAGLU	NM_000263	0.23	0.000
NBL1	NM_182744	0.11	0.000
NEK9	NM_033116	0.41	0.001
NF2	NM_181831	0.46	0.000
NPC1	NM_000271	0.34	0.000
OPTN	NM_001008211	0.04	0.000
P4HB	NM_000918	0.37	0.001
PALLD	NM_016081	0.29	0.001
PAPPA	NM_002581	0.05	0.000
PCDHGB4	NM_032098	0.28	0.001
PCK2	NM_004563	0.04	0.000
PCOLCE	NM_002593	0.00	0.000
PDGFRA	NM_006206	0.02	0.010
PEX14	BC017848	0.48	0.000
PFKL	NM_001002021	0.35	0.004
PPARG	NM_138711	0.01	0.000
PPFIBP2	NM_003621	0.08	0.000
PRR5	NM_015366	0.23	0.022
PSEN2	NM_012486	0.34	0.002
PTGS1	NM_000962	0.29	0.000
PXDN	AF200348	0.12	0.000
PYCARD	NM_013258	0.03	0.000
QSOX1	NM_002826	0.09	0.000
RASSF1	NM_170713	0.30	0.001
RBMS1	NM_002897	0.14	0.001
RECK	NM_021111	0.07	0.000
RET	NM_020975	0.35	0.015
RFPL1S	NR_002727	0.22	0.039
ROD1	NM_005156	0.37	0.001
RSU1	NM_012425	0.41	0.002
S100A4	NM_002961	0.03	0.000
SAMD9	NM_017654	0.07	0.007
SCARB2	NM_005506	0.42	0.001
SDC2	NM_002998	0.38	0.000
SDPR	NM_004657	0.03	0.005
SENP2	AF151697	0.44	0.006
SEPP1	NM_001085486	0.00	0.005
SFRP1	NM_003012	0.37	0.000
SHOC2	NM_007373	0.39	0.000
SIGIRR	NM_021805	0.47	0.000
SLC17A5	NM_012434	0.14	0.001
SLC22A5	NM_003060	0.21	0.001
SLC9A3R2	NM_004785	0.29	0.000
SMPD1	NM_000543	0.17	0.000
STAT1	NM_139266	0.19	0.000
STAT6	NM_003153	0.00	0.000
STS	NM_000351	0.10	0.007
STYK1	NM_018423	0.05	0.013
SUMF1	NM_182760	0.28	0.000
TAGLN	NM_001001522	0.01	0.000
TFAP2A	NM_003220	0.03	0.005
THBS2	NM_003247	0.02	0.000
THRA	NM_199334	0.31	0.000
THRB	NM_000461	0.10	0.014
TNXB	NM_019105	0.26	0.043
TPM2	NM_213674	0.12	0.000
TRIOBP	NM_007032	0.15	0.003
TRIP11	NM_004239	0.45	0.001
TSC22D3	NM_004089	0.14	0.000
TWIST1	NM_000474	0.01	0.003
VCAN	NM_004385	0.04	0.000
VCL	NM_014000	0.28	0.000
VLDLR	NM_003383	0.15	0.000
WISP1	NM_003882	0.05	0.013
WNT5A	NM_003392	0.01	0.000
YAP1	NM_006106	0.41	0.007
ZBTB7B	NM_015872	0.44	0.000

TABLE X5

Up-regulated Neural Precursor genes (NSLC vs. hNPC)			
GeneSymbol	Accession Number	Fold change of NSLC compared to hNPC[2]	p-value
ACTL6A	NM_178042	2.33	0.000
BCAT1	NM_005504	9.92	0.000
BDNF	NM_170735	33.90	0.000
BMP2	NM_001200	17.71	0.000
CDKN2A	NM_058197	5.57	0.000
COL18A1	NM_030582	7.22	0.001
DIAPH1	NM_005219	2.33	0.001
EDNRB	NM_003991	2.78	0.000
IDE	NM_004969	2.74	0.000
LIMK1	NM_002314	3.61	0.000
MAPK8IP1	NM_005456	2.77	0.000
MCHR1	NM_005297	4.02	0.000
MYLIP	NM_013262	4.22	0.000
NEDD4	NM_006154	2.23	0.000
NOS2A	NM_000625	267.58	0.000
PCSK9	NM_174936	9.65	0.000
PSEN2	NM_000447	2.07	0.000
SMAD1	NM_005900	3.09	0.000
TBX1	NM_080647	3.65	0.028
TGFB1	NM_000660	6.66	0.000

[2]Fold change represents the up-regulation of the gene in the NSLC samples as compared to the hNPC samples. (n = 3 for hNPC samples, n = 3 for NSLC samples).

TABLE X6

Up-regulated Glia genes (NSLC vs. hNPC)			
GeneSymbol	Accession Number	Fold change of NSLC compared to hNPC[1]	p-value
ACSL4	NM_004458	2.10	0.000
BDNF	NM_170735	33.90	0.000

TABLE X6-continued

Up-regulated Glia genes (NSLC vs. hNPC)			
GeneSymbol	Accession Number	Fold change of NSLC compared to hNPC[1]	p-value
BMP4	NM_001202	20.55	0.001
CP	NM_000096	159.46	0.000
CSPG4	NM_001897	4.94	0.000
FOXC1	NM_001453	5.12	0.000
GFAP	NM_002055	13.67	0.000
GJB2	NM_004004	7.25	0.000
GLIPR1	NM_006851	5.58	0.000
ITGA3	NM_002204	24.64	0.000
LMO3	NM_018640	129.25	0.000
NEFL	NM_006158	7.14	0.000
NKX6-2	NM_177400	11.50	0.000
NRTN	NM_004558	3.39	0.001
PDCD11	NM_014976	2.48	0.000
PLP1	NM_000533	20.64	0.000
TGFB1	NM_000660	6.66	0.000
TSPAN12	NM_012338	2.58	0.006

TABLE X7

Up-regulated Regenerative genes (NSLC vs. hNPC)			
GeneSymbol	Accession Number	Fold change of NSLC compared to hNPC[1]	p-value
ATR	NM_001184	2.57	0.000
BMP2	NM_001200	17.71	0.000
BMP4	NM_001202	20.55	0.001
CAV3	NM_001234	26.23	0.000
CCND1	NM_053056	10.34	0.000
CDKN2A	NM_058197	5.57	0.000
CEBPB	NM_005194	2.58	0.000
GAL	NM_015973	12.21	0.000
GAP43	NM_002045	4.27	0.000
HOXB4	NM_024015	133.37	0.000
SMAD3	NM_005902	2.27	0.000

In order to investigate the differentiation potential of NSLCs to neuronal lineages (Neurons, astrocytes, and oligodendocytes), neurospheres were dissociated and plated in laminin/poly-D-Lysine (10 μg/ml; Sigma) in differentiation medium for two weeks. The differentiation towards neuronal lineage was performed using two different mediums: NbActive medium (BrainBits™) supplemented with Brain Derived Neurotrophin Factor (BDNF, 20 ng/ml, Peprotech), all-trans-retinoic acid (ATRA, 5 μM, Spectrum), and bFGF (40 ng/ml, Peprotech) or NeuroCult™ differentiation medium (NeuroCult™ Differentiation kit, StemCell Technologies), supplemented with BDNF (20 ng/ml, Peprotech) and bFGF (40 ng/ml, Peprotech). After two weeks in culture, the cells were stained with the neuronal marker βIII-tubulin, astrocyte markers GFAP and S100β, and oligodendrocyte marker CNPase. The cells were fixed with 4% formaldehyde and the primary antibodies were added in 5% normal goat serum/PBS as follows: Mouse antibody βIII-tubulin (1:200, Abeam), rabbit antibody S100β (1:100, Abeam), and Chicken antibody CNPase (1:50, Abeam). Secondary antibodies are added in 5% normal goat serum/PBS as follows: Goat anti mouse Alexa546™ (1:200, Invitrogen). Goat anti rabbit Alexa488™ (1:200, Invitrogen), and Goat anti-chicken cy5 (1:100, Jackson ImmunoResearch Labs).

Immunohistochemistry analysis showed that NbActive medium promoted the differentiation equally to neuronal (48.66±14.07%, βIII-tubulin) and potential early oligodendrocyte lineages (50.01±4.04%, CNPase) and to a lower percentage of astrocyte cells (2.68±1.13%, S100β), while NS-A differentiation medium induced the differentiation mainly to neurons (64.89±4.11%, βIII-tubulin) and astrocytes (35.94±4.04%, S100beta), and a low percentage of potential early oligodendrocytes cells (8.68±2.71%, CNPase). The NSC-A medium was selected over NbActive for further differentiation studies. Differentiation of cells in NS-A differentiation medium promote the differentiation of hNPC and NSLC similarly as shown in Table 17 by the decrease of the percentage of sox2, musashi and nestin positive cells. NSLCs were differentiated to neuronal (74.3±0.1, GABA), astrocyte lineage (65.6±0.0, S100beta) and to a lower percentage of oligodendrocyte cells (5.2±0.6, CNPase). The same pattern of tripotent lineage differentiation was observed with hNPCs (Table 17).

TABLE 17

The percentage of cells stained positive for neural stem cell and neuronal lineage markers in transfected and untransfected cells. NSLCs and hNPCs were cultured in NS-A-differentiation medium supplemented with BDNF (20 ng/ml) and FGF (40 ng/ml), cultures were incubated at 37° C., 5% CO_2, 5% O_2 for three weeks. The percentage of immunopositive cells was determined by Cellomics™ and represented as mean ± SD (n = 5).

		Sox2	Nestin	Musashi	S100	O4	GABA
Tripotent medium	hNPC	73.8 ± 0.5	46.1 ± 5.2	22.1 ± 7.0	20.8 ± 1.3	6.4 ± 2.9	68.5 ± 1.6
	NSLC	68.6 ± 3.9	41.0 ± 5.4	26.7 ± 5.0	65.6 ± 0.0	8.2 ± 0.6	74.3 ± 0.1

Several additional antibodies to neuronal antigens were used to characterize, in more detail, the nature of differentiated cells. Antibodies against microtubule-associated protein (MAP2b), NCAM, and synaptophysin were used as recommended by the antibody manufacturer. After three weeks in differentiation medium, there was a differentiation-induced reduction in markers of precursors cells and an increase in mature neuronal markers. The percentage of neural precursor markers such as Sox2 were decreased during differentiation, while p75NTR, βIII-tubulin and GABA were increased with lengthening differentiation time; however, O4 positive cells were very low after 3 weeks of differentiation of hNPCs (6.4±2.9) and NSLCs (8.2±0.6). Synaptophysin, an antibody used to identify functional neuronal cells, was increased following 2 and 3 weeks of differentiation, indicating maturity of the neuronal cells. GABA and acetycholine markers were increased following 2 weeks of differentiation and decreased at week 3.

The morphological changes and expression of a number of neuronal antigens and genes show that the above method results in normal and viable neuronal cells. Additionally, the newly formed neuronal cells have the morphological criteria of neurons. In addition to the above markers, the differentiated cells were evaluated by characterizing morphological markers of neurite differentiation. Neuron type cells (cells strongly expressing βIII-tubulin) showed neurite formation after differentiation, including an increase in the average number of neurites per neuron (from e.g. 1.38±0.1) The same pattern was observed in βIII-tubulin positive cells. Accordingly, the average neurite length (118.3±3.5 μm) and the number of branch points (3.28±0.3) per neuron also increased. The differentiated neuron-like cells developed long neurites that were greater than three cell diameters in length with a growth cone at the end, expressed neuron-specific genes, and stopped proliferating after the induction of differentiation.

Further differentiation was performed using an optimised medium that promoted the differentiation towards oligodendrocyte lineage. NSLCs and hNPCs were cultured in NS-A differentiation medium as described previously supplemented with FGF-2 (10 ng/ml, Peprotech) and sonic hedgehog (SHH, 100 ng/ml, Peprotech) for 4 days. After 4 days medium was changed to NS-A differentiation medium supplemented by T3 (60 ng/ml, Peprotech), IGF1 (10 ng/ml, Peprotech), NT-3 (10 ng/ml, Peprotech), and PDGF (10 ng/ml, Peprotech). Cells were cultured for 20 days at 37° C., 5% CO_2.

TABLE 18

The percentage of cells stained positive for neural stem cell and neuronal lineage markers in transfected and untransfected cells. NSLCs and hNPCs were cultured in differentiation medium supplemented with SHH (100 ng/ml, Peprotech), T3 (60 ng/ml, Peprotech), IGF1 (10 ng/ml, Peprotech), NT-3 (10 ng/ml, Peprotech), and PDGF (10 ng/ml, Peprotech) to induce differentiation towards oligodendrocytes. The percentage of immunopositive cells was determined by Cellomics ™ and represented as mean ± SD (n = 5).

% of positive cells	Sox2	Nestin	Musashi	S100	O4	GABA
hNPC	84.3 ± 3.7	26.9 ± 4.4	51.8 ± 2.9	33.4 ± 1.9	40.1 ± 6.4	89.6 ± 0.8
NSLC	69.3 ± 4.4	24.3 ± 2.5	45.1 ± 11.1	51.6 ± 9.5	8.5 ± 0.6	76.9 ± 1.4

Quantification of the differentiation of hNPCs and NSLCs revealed a population of cells that were positively stained for O4. As shown in Table 18, the percentage of O4 positive cells was more pronounced in differentiated hNPC (40.1±6.4%) as compared to differentiated NSLCs (8.5±0.6%) when using the above differentiation protocol.

This study showed that transfecting the cells with one or two neurogenic transcription factors in the presence of a DNA demethylator or small molecules for epigenetic modification achieves stable reprogrammed cells (NSLCs). Like a DNA demethylator, epigenetic modification (inhibition of acetylation and methylation) are sometimes useful in boosting the reprogramming process. These cells possess and retain neural stem cell properties as determined by: (1) the expression of neural stem cell genes and proteins, (2) the capacity to generate and grow as neurospheres starting from a single cell, and (3) to differentiate to neuronal lineages in differentiation conditions. When differentiated to neurons, cells display one or more neural-specific morphological, physiological and/or immunological features associated with a neuronal cell type. Useful criteria include morphological features (long processes or neurites), physiological, and/or immunological features such as expression of a set of neuronal-specific markers or antigens. Furthermore, NSLCs readily turn into a tripotent-like precursor cell with differentiation potential to a high percentage of neuronal, astrocytes and lower percentage of oligodendrocyte populations.

Example VI

Implication of BMP Signaling Pathway in the Reprogramming of HFFs

This study was designed to evaluate the role of Noggin in the process of de-differentiation of HFFs towards NSLCs. HFFs were cultured and treated with cytochalasin B as described in Example III. After two days of treatment, cells were transfected by Nucleofection as described in Example II with the constructed vector Msi1/Ngn2. Briefly, after preparing the cells, they were mixed with 2 μg of total DNA (Msi1/Ngn2) and were co-transfected with MBD2 (2 μg), by the Amaxa's Nucleofector™ according to the manufacturer's protocol. The samples were then transferred into a Laminin (10 μg/ml, Sigma) coated culture plate and cultured in the presence of Neural Proliferation Medium (NeuroCult™ proliferation Kit, StemCell Technologies) with recombinant hFGF (20 ng/ml, Peprotech), recombinant hEGF (20 ng/ml, Peprotech), and with or without the presence of Noggin (20 ng/ml, Peprotech). Samples were collected at different time points (1, 3, 4, 6, and 8 days) to analyze neuronal gene expression by RT-PCR and protein expression levels by immunohistochemistry.

Fluorescent immunohistochemical staining was performed on samples after 4 days of transfection as previously described in Example I. Transfected cells were stained and analyzed for expression of Sox2, the percentage of Sox2 was 33.3±1.00% in the presence of Noggin compared to 27.5±0.50% without the presence of noggin at day 4. RT-PCR analysis of relative expression of neuronal precursor cell markers such as nestin and Sox2 after transfection of HFFs with pCMV-Msi1-2A-Ngn2 and pCMV6-XL5-MBD2 with or without the presence of Noggin (20 ng/ml) was associated with an increase in nestin and Sox2 starting at day 3 and maintained until day 8 (Table 19). No difference in the expression was noticed in the absence of Noggin. Inhibiting the BMP signaling pathway by Noggin thus enhanced reprogramming, but had no reprogramming effect on its own.

TABLE 19

RT-PCR analysis of relative expression of neuronal precursor cell markers such as nestin and Sox2 after transfection of HFF with pCMV-Msi1-2A-Ngn2 and pCMV6-XL5-MBD2 with or without Noggin (20 ng/ml). Relative expression of Sox2, and nestin was increased after transfection with and without Noggin.

	ACHE		GFAP		NES		SOX2		TUBB3	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1 Msi1/Ngn2 + MBD2/+Noggin Day 1	7.08	1.70	2.97	0.42	1.33	0.10	0.93	0.91	1.37	0.10
#2 Msi1/Ngn2 + MBD2/+Noggin Day 2	7.34	1.03	2.01	0.08	1.28	0.18	0.60	0.10	0.98	0.05
#3 Msi1/Ngn2 + MBD2/+Noggin Day 3	9.67	2.41	15.13	1.66	1.98	0.20	6333.63	277.87	0.95	0.07
#4 Msi1/Ngn2 + MBD2/+Noggin Day 4	11.68	2.65	194.07	25.22	4.19	0.52	20231.33	1034.29	1.90	0.45
#5 Msi1/Ngn2 + MBD2/+Noggin Day 6	3.58	0.66	227.99	16.83	1.68	0.09	6298.51	289.84	0.96	0.17
#6 Msi1/Ngn2 + MBD2/+Noggin Day 8	10.89	0.57	650.34	22.92	4.42	0.03	18134.90	63.93	1.81	0.06
#7 Ctrl Untransfected + Noggin Day 1	1.01	0.19	1.00	0.05	1.00	0.02	1.12	0.70	1.00	0.09
#8 Msi1/Ngn2 + MBD2/ −Noggin Day 1	2.79	0.83	1.62	0.19	0.99	0.08	1.28	0.25	0.75	0.01
#9 Msi1/Ngn2 + MBD2/ −Noggin Day 2	3.79	0.91	1.47	0.08	1.23	0.08	1.36	0.08	0.72	0.07
#10 Msi1/Ngn2 + MBD2/ −Noggin Day 3	6.18	0.59	14.60	1.85	2.62	0.30	10949.28	448.28	0.90	0.01
#11 Msi1/Ngn2 + MBD2/ −Noggin Day 4	5.63	0.74	74.56	16.56	2.97	0.21	19623.99	3109.69	0.75	0.11
#12 Msi1/Ngn2 + MBD2/ −Noggin Day 6	3.21	0.96	232.42	5.47	1.47	0.07	15311.64	1909.23	0.86	0.03
#13 Msi1/Ngn2 + MBD2/ −Noggin Day 8	3.82	0.52	496.99	75.81	3.32	0.32	26892.31	1817.05	2.05	0.10
#14 Ctrl Untransfected − Noggin Day 1	1.08	0.57	1.01	0.14	1.00	0.04	1.15	0.81	1.00	0.00

Example VII

NSLCs Created from DIFF Cells are not Skin-Derived Precursors (SKPs)

Its known that cells termed skin-derived precursors (SKPs) may reside in adult human skin (Fernandes et al., 2004). These cells are capable of proliferating in response to EGF and bFGF and express nestin, versican and fibronectin, and can differentiate into both neuronal and mesodermal progeny. In order to verify that NSLCs are distinct from SKPs, differentiation towards adipocyte cells was performed. Adipose derived stem cells (ADSC) were maintained in StemPro™ MSC serum free medium (Invitrogen) on flasks coated with CellStart™ (Invitrogen). CellStart™ was diluted 1:100 in dPBS/Ca^{2+}/Mg^{2+} and the flask incubated for 2 hours at 37° C. Cells are passaged every 3 to 4 days using Accutase™ and medium was changed every 2 days. Three to four days before initiating differentiation, ADSCs and NSLCs were seeded in 6-wellplates in CellStart™ (1:100 in dPBS/Ca^{2+}/Mg^{2+}/2 hours at 37° C.) coated tissue culture plates. When cells reached confluence (after 3 to 4 days), proliferation media were replaced by differentiation medium consisting in DMEM/F12 (50:50), ITS (1:100), HEPES (1:100), GlutaMAX™ (1:100), T3 (0.2 nM), Rosiglitasone (0.5 μg/rap, IBMX (100 μM) and Dexamethasone (1 μM). Three days after, IBMX and dexamethasone were withdrawn from the differentiation medium. At day 10, cells were fixed with a 4% formaldehyde solution for 10 min and stained with Oil Red O (Invitrogen) staining solution for 15 min. Staining was removed and cells washed twice with PBS. Adipose cells appeared red with lipid droplets specifically stained with Oil Red O, however NSLCs were stained negative, with no presence of lipid droplets in the cells, and the cells adopted neuronal cell morphology.

Immunohistochemistry analysis confirmed that NSLCs are distinct from SKPs: NSLCs stained positive for p75NTR and negative for fibronectin and versican, while SKPs express fibronectin and versican and do not express p75NTR (Fernandes et al., 2004). This study indicates that NSLCs represent a tripotent-like precursor cell and they are not a subpopulation of SKPs.

Example VIII

BDNF Release from Neural-Like Cells (NLCs)

Neural Stem-Like Cells (NSLCs) differentiated into neuronal and glial cells were kept in culture for 55 days, and BDNF released in the conditioned medium was measured by antigen-capture ELISA at different time points and compared to the release in mature neurons (ScienCell), undifferentiated Neural Human Normal Precursor cells (NRNP, Lonza) as well as to undifferentiated NSLCs and untransfected cells (HFF). Conditioned medium from each group was collected, centrifuged, and then stored at −80° C. until assaying. BDNF concentrations were measured by ELISA kits (BDNF E_{max} Immunoassay System, Promega Corporation, USA), according to the manufacturer's instructions. Briefly, 96-well ELISA immunoplates were coated with Anti-BDNF (CatNb#G700B) diluted 1/1000 in carbonate buffer (pH 9.7) and incubated at 4° C. overnight. The following day, all wells were washed with TBS-Tween™ 0.5% before incubation with Block/Sample buffer 1× at room temperature for one hour without shaking. After blocking, standards and samples were added to the plates and incubated and shaken (450±100 rpm) for 2 h at room temperature. Subsequently, after washing with TBS-Tween™ wash buffer, plates were incubated for 2 h with Anti-Human BDNF pAb (1:500 dilution in Block & Sample 1× Buffer) at 4° C. After incubation, plates were washed five times with TBS-Tween™ 0.5% wash buffer and 100 μl of diluted Anti-IgYHRP Conjugate was added to each well (1:200 dilution in Block & Sample 1× Buffer) and incubated for 1 hour at room temperature with shaking (450±100 rpm). Then, plates were washed five times with TBS-Tween™ 0.5% wash buffer and 100 μl of TMB One Solution was added to each well. Following 10 minutes incubation at room temperature with shaking (450±100 rpm) for the BDNF plate, a blue color formed in the wells. After stopping the reaction by adding 1000 of 1N hydrochloric acid, the absorbance was read at 450 nm on a microplate reader (Synergy 4™) within 30 minutes of stopping the reactions. Concentration of released BDNF in the supernatants was determined according to the standard curves.

ELISA results revealed that BDNF was released at the same concentration from differentiated Neuron-Like Cells (NLCs differentiated from NSLCs) and normal Human neuron cells starting at day 11 and remained until day 55 (Table 20), while no BDNF (except for tiny amounts in the untransfected HFF group) was released in the other groups.

TABLE 20

Quantification of BDNF release by Neural-Like Cells (NLCs) that had been differentiated for 55 days from Neural Stem-Like Cells (NSLCs) that had been created from transfected HFFs. BDNF release from NLCs into the medium, at different time points, was measured by antigen-capture ELISA and compared to BDNF release of normal mature human neurons (ScienCell).

	Control medium	Neurons	NLC
day 0			
day 11	1.55	30.25	22.99
day 18	0.33	29.49	25.15
day 24	0.33	22.01	26.39
day 34	0.23	25.53	32.21
day 41	0.27	19.02	22.43
day 55	0.02	20.73	30.01

In addition to adopting neuronal morphology criteria, the NLCs were functional and possessed the capacity to release neurotrophic factor (BDNF). Generating reprogrammed neuronal-like cell lines that can locally deliver these neurotrophic factors could be used as a method to treat several neurological conditions and may offer crucial benefits in regeneration and functional recovery from brain and other injuries.

Example IX

Reprogramming of Different Cell Types Towards NSLCs

This study was performed to investigate the capacity of keratinocytes (Invitrogen), human Adipocytes Derived Stem Cells (ADSCs, Invitrogen) and human hematopoietic stem cells ($CD34^+$, Invitrogen) cells into neural stem-like cells.

Preparation of Human $CD34^+$ Cells, Human ADSC and Human Keratinocytes:

Human mobilized peripheral blood $CD34^+$ cells were purchased from StemCell Technologies and expanded as a floating culture in Petri Dishes in complete StemPro™-34 Serum-free Medium (Invitrogen) supplemented with Stem Cell Factor (SCF, 150 g/ml, Peprotech), Granulocyte Colony-Stimulating Factor (GM-CSF, 37.5 ng/ml, Peprotech) and IL-3 (75 ng/ml, Peprotech). Medium supplemented with cytokines was changed everyday 2-3 days after centrifugation of the cell suspension at 300×g for 10 min. Every other day the cytokines were added directly to the culture without changing the media. Cells were incubated at 37° C., 5% CO_2. For their passaging, cells were centrifugated, resuspended in the above medium plus cytokines and placed into the adequate number of Petri dishes.

Human Adipose-Derived Stem Cells (ADSC) were purchased from Invitrogen and expanded in complete StemPro™ MSC Serum-free medium (Invitrogen) on CellStart™™ (Invitrogen) coated flasks (diluted 1:100 in PBS containing Ca^{2+}/Mg^{2+}) at a cell density of 1×10^4 cells/cm^2. Medium was replaced every two days with fresh pre-warmed complete StemPro™ MSC SFM. Cells were incubated at 37° C., 5% CO_2. Cells were sub-passaged when 80% confluent by incubation for 3-5 min in pre-warmed TrypLE™™ (Invitrogen) and then collected in StemPro™ MSC medium. After centrifugation at 1500 rpm for 5 min, cells were seeded on CellStart™ coated flasks as described above.

Primary human keratinocytes were purchased from Invitrogen and expanded in Defined Keratinocyte Serum-free medium on Coating matrix (Invitrogen) coated flasks (Invitrogen) at a cell density of 5×10^3 cells/cm^2. The cells were incubated at 37° C., 5% CO_2. Media was replaced with fresh, complete growth media every two to three days until subculture. Once the cells had reached 70-80% confluency, media was removed and the cells were incubated in Versene™ (Invitrogen) for 3-5 min at room temperature. Versene™ was removed, and pre-warmed 0.05% trypsin-EDTA (Invitrogen) was added to the flasks. After 5-10 min incubation, growth medium containing Soybean Trypsin inhibitor (Invitrogen) was added to the flasks and the cells gently triturated. After centrifugation at 100×g for 10 min, cells were resuspended in the desired volume of pre-warmed, complete growth medium on coated flasks as described above.

Prior to transfection, cells were trypsinized and transiently co-transfected with pCMV-Msi1-Ngn2 and pCMV6-XL5-MBD2 as previously described in Example IV using the Shuttle and plated into a culture plate coated with laminin (Sigma, 10 μg/ml). Starting one day after transfection, cells were treated with VPA (1 mM) for 4 days and the medium was changed gradually to proliferation medium supplemented with FGF (20 ng/ml) and EGF (20 ng/ml) and were cultured for 18 days at 37° C., 5% CO_2 and 5% O_2. Cells were then analyzed for neural stem cell markers by RT-PCR and Immunohistochemistry.

Further analysis and quantification of the reprogrammed cells revealed a population of NSLCs engendered from keratinocyte and CD34$^+$ cells. RT-PCR Analysis revealed an increase of relative expression of neural stem cell markers such as Sox2, nestin, GFAP, and βIII-tubulin after transfecting Keratinocyte and CD34$^+$ by Msi1 and Ngn2. Relative expression of nestin and GFAP was enhanced in NSLCs created from keratinocytes and CD34$^+$ cells as compared to NSLCs from HFFs; however, the reverse was true for Sox2 and ACHE expression. βIII-tubulin (TUBB3) and Map2b expression was highest in NSLCs created from CD34$^+$ cells, followed by NSLCs created from HFF (Table 21). This data shows that different types of NSLCs with different gene expression profiles (and characteristics) can be created from different types of starting/source cells (and the same has been observed for creating some other types of stem-like cells discussed in this application). The data is also intriguing since it was not expected that keratinocytes (which are derived from the ectoderm just as endogenous neural stem cells) would have a lower expression than HFFs for all the genes analyzed except for Nestin (it was expected that keratinocytes would be the easiest to reprogram into NSLCs since they are derived from the ectoderm).

TABLE 21

RT-PCR analysis was performed after one month of transfection of human fibroblasts (HFF), Keratinocytes, and CD34$^+$ cells with Msi1/Ngn2 (MSI1/NGN2), in the presence MBD2 with VPA treatment. Cells were cultured on coated culture plates in proliferation medium (StemCell Technologies) supplemented with EGF (20 ng/ml) and FGF (20 ng/ml) for 18 days. Untransfected cells were considered as negative control.

	NES		MAP2		TUBB3		ACHE		GFAP		SOX2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1 Day 12 Untransfected HFF	1.00	0.07	1.00	0.05	1.00	0.01	1.01	0.15	1.00	0.02	1.08	0.59
#2 Day 12 HFF Msi1/Ngn2 + MBD2	2.25	0.03	21.48	2.09	3.41	0.45	12.92	1.88	558.69	80.08	71513.12	14146.80
#3 Day 18 HFF Msi1/Ngn2 + MBD2	2.56	0.15	17.12	0.14	2.65	0.02	4.13	0.64	75.96	8.82	84794.40	318.54
#4 Untransfected Keratinocytes	1.07	0.54	1.00	0.07	1.00	0.02	1.01	0.19	1.06	0.48	1.00	0.01
#5 Day 12 Keratinocytes Msi1/Ngn2 + MBD2	11452.65	1137.13	0.96	0.11	6.78	0.28	1.09	0.05	5815.54	510.91	975.81	7.47
#6 Day 18 Keratinocytes Msi1/Ngn2 + MBD2	12593.79	431.06	0.93	0.04	6.41	0.27	0.48	0.03	1295.15	32.05	1047.17	139.48
#7 Untransfected CD34+	1.00	0.04	1.01	0.16	1.00	0.00	1.00	0.01	1.10	0.66	1.01	0.21
#8 Day 18 CD34+ Msi1/Ngn2 + MBD2	839.57	134.51	346.61	33.97	33.91	4.38	0.28	0.00	2790.18	304.43	25080.35	35.93
hNPC	4.56	0.07	278.36	11.50	0.81	0.06	72.65	1.83	1285.73	5.27	565552.30	41717.72

Immunohistochemistry revealed positive staining for GFAP, Sox2, and nestin. NSLCs developed from HFF yield a higher percentage of positive staining for Sox2 and GFAP (55.8±3.8 and 78.1±2.4) as compared to $CD34^+$ cells (42.8±2.7 and 24.2±4.4), and keratinocytes (47.1±2.1 and 43.4±89). The percentage of nestin positive cells was high in Keratinocytes (77.6±10.7) and HFF (68.45±12.9) and lower in $CD34^+$ cells (155±2.7) (Table 22). Sox2 and Nestin positive staining was undetectable in ADSCs.

TABLE 22

The percentage of Sox2 and nestin positive cells for neural stem cell markers after transfecting fibroblast, keratinocyte, and $CD34^+$ cells with pCMV-Msi1-Ngn2 in the presence of MBD2 and VPA. Cells were cultured on coated culture plates in proliferation medium (StemCell Technologies) supplemented with EGF (20 ng/ml) and FGF (20 ng/ml) for 18 days. Untransfected cells were considered as negative control. The percentage of immunopositive cells was determined by Cellomics ™ and represented as mean ± SD (n = 5).

% positive cells	Untransfected cells	Fibroblasts	Keratinocytes	CD34+
Sox2	1.5 ± 1.7	55.8 ± 3.8	47.1 ± 2.1	42.8 ± 2.7
GFAP	0.04 +/− 0.2	78.1 ± 2.4	43.4 ± 8.9	24.2 ± 4.4
Nestin	0.3 +/− 0.3	68.45 ± 12.9	77.6 ± 10.7	15.5 ± 2.7

NSLCs generated from keratinocytes and $CD34^+$ cells were tested for tripotent capacity. Further differentiation studies were performed to induce differentiation of these NSLCs towards neuronal lineage, using NeuroCult™ differentiation medium (NeuroCult™ differentiation Kit, StemCell Technologies) supplemented with BDNF (20 ng/ml, Peprotech) and bFGF (40 ng/ml, Peprotech) as described in Example V. NSLCs generated from HFFs and hNPCs were used as controls, cultures were incubated at 37° C., 5% CO_2, 5% O_2 for three weeks. Samples were collected or fixed at Day 14 and 28 following differentiation for further analysis. RT-PCR analysis revealed decrease of undifferentiated genes (Nestin and Sox2) and increased of differentiated genes (Map2, βIII-tubulin, CNPase, and GFAP) as shown in Tables 23A, 23B, 23C and 23D.

TABLE 23A

RT-PCR analysis was performed on NSLCs generated from human fibroblasts (HFF), keratinocytes, and $CD34^+$ cells that were cultured on Poly-D-Lysin/Laminin coated culture plates in differentiation medium for 28 days (StemCell Technologies) supplemented with BDNF (20 ng/ml) and FGF (40 ng/ml). hNPCs (Lonza) were considered as a positive control. hNPCs had a much lower increase in ACHE, GFAP, and MAP2b (which actually decreased in hNPCs), but an increase in Nestin, compared to NSLCs under differentiation conditions.

	NES		MAP2		TUBB3		ACHE		GFAP		SOX2		SOX9		CNP	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
hNPC Control	1.00	0.08	1.00	0.10	1.00	0.08	1.01	0.16	1.00	0.09	1.01	0.16	1.00	0.12	1.00	0.09
Diff. hNPC Day 14	3.86	0.20	0.65	0.05	4.87	0.57	0.74	0.52	97.26	7.13	1.85	0.21	0.50	0.04	1.43	0.05
Diff. hNPC Day 28	1.86	0.06	0.68	0.02	3.67	0.13	1.33	0.09	102.74	1.89	1.29	0.01	0.73	0.05	1.37	0.02
NSLC Control	1.00	0.04	1.00	0.04	1.00	0.04	1.00	0.03	1.00	0.01	1.00	0.01	1.00	0.02	1.00	0.05
Diff. NSLC Day 14	1.38	0.01	1.00	0.09	2.06	0.02	1.57	0.24	1.79	0.12	0.73	0.01	0.56	0.01	1.31	0.05
Diff. NSLC Day 28	0.62	0.02	0.90	0.08	5.14	0.21	6.47	0.78	5.70	0.15	1.30	0.02	0.79	0.03	1.41	0.01
HFF-NS Control	1.00	0.00	1.00	0.05	1.00	0.01	1.00	0.07	1.00	0.00	1.00	0.07	1.00	0.01	1.00	0.02
Diff. HFF-NS Day 14	2.70	0.08	3.08	0.12	3.24	0.14	59.93	5.85	478.97	0.27	2.90	0.32	0.81	0.03	4.02	0.35
Diff. HFF-NS Day 28	1.27	0.05	1.48	0.11	1.59	0.03	24.62	1.00	576.80	20.98	1.52	0.00	0.86	0.08	2.74	0.23
Kerat-NS Control	1.00	0.06	1.00	0.02	1.00	0.03	1.00	0.11	1.00	0.01	1.00	0.07	1.00	0.02	1.00	0.01
Diff. Kerat-NS Day 14	2.43	0.06	3.48	0.08	2.82	0.11	56.22	5.58	665.91	10.52	3.09	0.29	1.01	0.14	3.72	0.17
Diff. Kerat-NS Day 28	0.81	0.03	1.72	0.00	1.61	0.18	26.09	1.12	673.65	11.34	1.29	0.03	1.12	0.03	2.02	0.05
CD34+-NS Control	1.00	0.05	1.00	0.07	1.00	0.04	1.00	0.08	1.00	0.00	1.00	0.08	1.00	0.02	1.00	0.07
Diff. CD34+-NS Day 14	2.21	0.04	3.47	0.07	2.75	0.04	57.87	6.68	407.54	52.07	2.90	0.18	1.10	0.05	3.54	0.02
Diff. CD34+-NS Day 28	0.79	0.04	1.48	0.01	1.83	0.37	26.92	3.73	485.51	10.66	1.02	0.04	1.20	0.09	2.34	0.05

TABLE 23B

RT-PCR analysis was performed on undifferentiated NSLCs generated from human fibroblasts (HFF), keratinocytes, and CD34+ cells that were cultured on Laminin coated culture plates in Proliferation medium for 4 days (StemCell Technologies) supplemented with EGF (20 ng/ml) and FGF (20 ng/ml). Relative expression calibrated to undifferentiated hNPCs.

	NES		MAP2		TUBB3		ACHE		GFAP		SOX2		SOX9		CNP	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
Undifferentiated hNPC Control Day 4	1.00	0.08	1.00	0.10	1.00	0.08	1.01	0.16	1.00	0.09	1.01	0.16	1.00	0.12	1.00	0.09
Undifferentiated NSLC Control Day 4	1.23	0.05	0.12	0.00	1.12	0.04	0.09	0.00	21.45	0.26	0.65	0.01	0.28	0.01	0.37	0.02
Undifferentiated HFF-NS Control Day 4	0.94	0.00	0.12	0.01	0.92	0.01	0.03	0.00	0.38	0.00	0.37	0.02	0.32	0.00	0.31	0.00
Undifferentiated Kerat-NS Control Day 4	1.00	0.06	0.09	0.00	0.97	0.03	0.03	0.00	0.23	0.00	0.38	0.03	0.26	0.00	0.30	0.00
Undifferentiated CD34+-NS Control Day 4	1.10	0.05	0.12	0.01	0.95	0.04	0.04	0.00	0.33	0.00	0.44	0.04	0.26	0.00	0.30	0.02

TABLE 23C

RT-PCR analysis was performed on differentiated NSLCs generated from human fibroblasts (HFF), keratinocytes, and CD34+ cells that were cultured on Poly-D-Lysin/Laminin coated culture plates in differentiation medium for 14 days (StemCell Technologies) supplemented BDNF (20 ng/ml) and FGF (40 ng/ml). Relative expression calibrated to Day 14 differentiated hNPCs.

	NES		MAP2		TUBB3		ACHE		GFAP		SOX2		SOX9		CNP	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
Diff. hNPC Day 14	1.00	0.05	1.00	0.07	1.00	0.12	1.15	0.80	1.00	0.07	1.00	0.11	1.00	0.08	1.00	0.03
Diff. NSLC Day 14	0.44	0.00	0.18	0.02	0.47	0.00	0.22	0.03	0.40	0.03	0.26	0.00	0.31	0.00	0.34	0.01
Diff. HFF-NS Day 14	0.66	0.02	0.56	0.02	0.62	0.03	2.96	0.29	1.86	0.00	0.58	0.06	0.52	0.02	0.87	0.08
Diff. Kerat-NS Day 14	0.63	0.02	0.51	0.01	0.56	0.02	2.78	0.28	1.56	0.02	0.64	0.06	0.54	0.08	0.79	0.04
Diff. CD34+-NS Day 14	0.63	0.01	0.62	0.01	0.54	0.01	3.77	0.43	1.39	0.18	0.69	0.04	0.58	0.03	0.76	0.00

TABLE 23D

RT-PCR analysis was performed on differentiated NSLCs generated from human fibroblasts (HFF), keratinocytes, and CD34+ cells that were cultured on Poly D-Lysin/Laminin coated culture plates in differentiation medium for 28 days (StemCell Technologies) supplemented with BDNF (20 ng/ml) and FGF (40 ng/ml). Relative expression calibrated to Day 28 differentiated hNPCs.

	NES		MAP2		TUBB3		ACHE		GFAP		SOX2		SOX9		CNP	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
Diff. hNPC Day 28	1.00	0.03	1.00	0.02	1.00	0.04	1.00	0.07	1.00	0.02	1.00	0.01	1.00	0.07	1.00	0.02
Diff. NSLC Day 28	0.41	0.01	0.15	0.01	1.56	0.06	0.44	0.05	1.19	0.03	0.66	0.01	0.30	0.01	0.38	0.00
Diff. HFF-NS Day 28	0.64	0.03	0.26	0.02	0.40	0.01	0.59	0.02	2.12	0.08	0.43	0.00	0.38	0.04	0.62	0.05
Diff. Kerat-NS Day 28	0.44	0.02	0.24	0.00	0.42	0.05	0.62	0.03	1.50	0.03	0.38	0.01	0.40	0.01	0.44	0.01
Diff. CD34+-NS Day 28	0.47	0.03	0.25	0.00	0.47	0.10	0.85	0.12	1.57	0.03	0.35	0.01	0.43	0.03	0.52	0.01

Fluorescent immunohistochemical staining was performed on samples after 14 days and 28 days of differentiation. The expression of Sox2 and Nestin was decreased time dependently in differentiated cells (HFF, keratinocyte, and $CD34^+$). This decrease was associated with an increase of differentiated markers at day 28 such as GFAP (68.51±11.87 for HFF-NC, 59.55±9.12 for Keratinocyte NC, and 61.70±1.48 for $CD34^+$-NC). A high percentage for βIII-tubulin positive cells was generated from differentiated NSLCs generated from HFF (57.83±4.49) as compared to βIII-tubulin positive cells generated from Keratinocytes (23.27±2.91) and $CD34^+$ cells (39.15±7.99) (Table 24)

TABLE 24

The percentage of cells stained positive for neural stem cell markers and neuronal lineage markers in hNPCs (Lonza) and transfected keratinocytes, HFF, and $CD34^+$ cells with pMsi1/Ngn2/MBD2. Transfected cells (NSLCs) were cultured in Proliferation medium or differentiation medium for 28 days at 37° C., 5% CO_2, 5% O_2. The percentage of immunopositive cells (Sox2, Nestin, GFAP, S100beta, and βIII-tubulin) was determined by Cellomics ™ and represented as mean ± SD (n = 5)

	% positive cells	Proliferation conditions	14 days differentiation	28 days differentiation
hNPC	Sox2	96.23 ± 0.51	59.05 ± 3.01	41.43 ± 6.05
	Nestin	41.47 ± 0.23	10.77 ± 4.78	16.14 ± 7.41
	s100β	37.38 ± 7.85	49.51 ± 2.39	n.d.
	βIII-tubulin	2.34 ± 0.43	11.54 ± 4.03	23.34 ± 4.77
	GFAP	1.16 ± 0.14	23.42 ± 2.51	48.04 ± 8.30
HFF-NC	Sox2	93.28 ± 0.53	79.48 ± 0.54	52.06 ± 9.07
	Nestin	29.29 ± 4.72	1.15 ± 0.46	2.18 ± 1.96
	s100β	13.51 ± 0.28	80.75 ± 3.50	79.38 ± 10.62
	βIII-tubulin	3.91 ± 0.33	42.16 ± 15.07	57.83 ± 4.49
	GFAP	8.41 ± 0.73	59.66 ± 11.48	68.51 ± 11.87
Keratinocyte-NC	Sox2	96.55 ± 1.01	76.93 ± 5.13	63.11 ± 8.54
	Nestin	40.10 ± 8.41	2.67 ± 1.61	3.57 ± 0.48
	s100β	13.58 ± 4.97	76.6 ± 9.72	74.75 ± 11.21
	βIII-tubulin	6.42 ± 2.94	20.58 ± 8.34	23.27 ± 2.91
	GFAP	9.36 ± 0.34	43.43 ± 2.44	59.55 ± 9.12
$CD34^+$-NC	Sox2	95.49 ± 2.6	81.18 ± 1.24	63.46 ± 5.14
	Nestin	51.68 ± 14.27	12.64 ± 1.27	8.46 ± 4.6
	s100β	30.1 ± 1.03	72.40 ± 4.5	79.57 ± 8.52
	βIII-tubulin	5.82 ± 2.08	25.04 ± 19.95	39.15 ± 7.99
	GFAP	13.99 ± 5.48	51.79 ± 13.68	61.70 ± 1.48

n.d. = not determined; ± = standard deviation

$CD34^+$-NC: neuronal cells generated after differentiation of NSLCs generated from $CD34^+$ cells. Each data point represents the analysis of at least 1000 cells from at least 8 images.

The % of Sox2 positive cells decreased faster, the % of Nestin positive cells generally decreased slower, and the % of cells expressing one of the differentiation markers (S100β, βIII-tubulin, GFAP) generally increased slower in hNPCs than in the NSLCs during differentiation. Out of the three types of created NSLC lines, the % of cells expressing one of the differentiation markers (S100β, βIII-tubulin, GFAP) generally increased slowest in NSLCs created from keratinocytes and fastest in NSLCs created from HFFs.

This study indicates that NSLCs can be created from keratinocytes and $CD34^+$ blood cells, and these cells share morphology and markers similarly to NSLCs generated from HFF. Similarly to hNPCs, NSLCs created from keratinocytes, $CD34^+$ cells, and HFFs had a tendency to differentiate more towards an astrocyte lineage than a neuronal lineage (except NSLCs created from HFFs had an almost similar number of βIII-tubulin positive and GFAP positive cells) as shown by the high percentage of GFAP positive cells during differentiation, which was confirmed by S100beta staining. However, the proportion of astrocyte and neuronal cells generated from hNPCs was lower in same culture conditions, indicating that NSLCs generated from HFF, Keratinocytes, and $CD34^+$ cells can give rise to a higher number of neuronal and astrocyte cells as compared to hNPCs. NSLCs, whether created from HFFs, Keratinocytes or $CD34^+$ cells (or potentially even some other cell), are tripotent cells and possess the capacity to differentiate to neurons, astrocytes, and oligodendrocytes similarly to hNPCs. However, RT-PCR and immunohistochemistry analysis of transfected ADSCs did not reveal any significant expression of neural stem cell genes, indicating a need to optimize conditions for turning ADSCs to NSLCs or to investigate the effect of others neurogenic factors that could turn these into NSLCs.

Example X

Fabrication 3D Extracellular Matrix (CDM)

Fibroblast cells were cultured in DMEM medium in the presence of 10% FCS as described in Example I, followed by seeding onto 12-well plates pre-coated with laminin (10 μg/ml) at a concentration of 2×10^6 cells/ml in defined CDM Medium consisting of a 3:1 ratio of Dulbecco's modified Eagle medium (DMEM, high glucose (4.5 g/L) with L-glutamine and sodium pyruvate) and Ham's F-12 medium supplemented with the following components: EGF (4.2×10^{-10}M), bFGF (2.8×10^{-10} M), ITS (8.6×10^{-5}M), dexamethasone (1.0×10^{-7}M), L-ascorbic acid phosphate magnesium salt n-hydrate (3.2×10^{4}M), L-3,3',5-triiodothyronine (2.0×10^{-10}M), ethanolamine (10^{-4}M), GlutaMAX™ (4×10^{-3}M), glutathione (3.3×10^{-6}M), and 1% penicillin/streptomycin/amphotericin B. By culturing the fibroblast cells at hyperconfluent density in this completely chemically defined medium causes them to enter a high synthetic phase with a slow-down in proliferation, leading to the production of a living tissue equivalent (LTE) consisting of multiple layers of fibroblasts within de novo 3D extracellular matrix (CDM) that is completely synthesized by the fibroblasts themselves.

Trans-Differentiation and Reprogramming of Cells within CDM

Day 14 CDM samples were treated with cytochalsin B (10 μg/ml, Calbiochem), with the concentration of cytochalsin B reduced from 10 μg/ml to 0 μg/ml (none) over 5 days while at the same time switching the medium from CDM Medium to NbActive medium. Samples were cultured for another 12 days at 37° C., 5% CO_2, and the medium was changed every day. Samples were fixed to perform immunohistochemistry as described previously to detect Neuronal markers. The following antibodies were used: mouse anti-nestin 647 (1:100, BD) and anti-βIII-tubulin (1:200, Neuromics). No clear morphology change of the cells was observed within the CDM and the immunohistochemical analysis failed to detect βIII-tubulin positive cells. Thus, inducing the trans-differentiation of cells using only cytochalasin. B and chemically-defined neural medium was not sufficient to reprogram the cells.

Next, Day 6 CDM samples grown in LAS pre-coated plates at 37° C. and 5% CO_2, were exposed simultaneously to cytocahlasin B (10 μg/ml) over 5 days, histone deacetylation inhibitor (VPA, 4 mM, Calbiochem) and inhibitor of DNA methylation (5-Azacytidine, 5 μM, Sigma). Four days later, the medium was changed to differentiation medium consisting of a 3:1 ratio of CDM medium without the presence of EGF and NbActive medium (BrainBits™) supplemented with NT-3 (20 ng/ml, Peprotech) and BDNF (20 ng/ml, Peprotech). The ratio of the differentiation medium was increased gradually day after day until reaching 100% of complete differentiation medium. After two weeks of treatment, cells were fixed for immunohistochemical analysis to investigate the identity of the cells. Immunostained cells with βIII-tubulin at day 7, indicated the de-differentiation of fibroblast cells to neurons. However, one week later, these transdifferentiated cells reverted back to fibroblast cells and βIII-tubulin expression was lost. The loss

of morphology and βIII-tubulin expression after withdrawal of the priming agents indicate that complete conversion to functional and stable reprogrammed cells did not occur.

Next CDM was treated with VPA (4 mM), 5-Aza (5 μM) and cytochalasin B (10 μg/ml) as above. After 2 days of chemical treatment, fibroblast cells within the CDM were transfected with DNA using Lipofectamine reagent (Invitrogen) as per the manufacturer's protocol. 15 μg of the eukaryotic DNA expression vectors pCMV6-XL5-Pax6, pCMV6-XL5-Msi1 and pCMV6-XL4-Ngn2 (Origene) were used to transfect the cells. 24 hours later, the media was changed to Neural Progenitor Basal Medium (Lonza) supplemented with Noggin (50 ng/ml), EGF (20 ng/ml), and bFGF (20 ng/ml), and the cells were cultured at 37° C., 5% $CO_2$and 5% O_2, and the medium was changed every day. At day 6, differentiation was initiated by adding gradually NBActive medium (BrainBits™) supplemented with NT-3 (20 ng/ml, Peprotech), all-trans-retinoic acid (ATRA, 5 μM, Spectrum), BDNF (20 ng/ml, Peprotech), and bFGF (40 ng/ml, Peprotech). To characterize the reprogrammed cells, immunohistochemical analysis and RT-PCR was performed at various time points according to the methods described in Example II using primers for nestin, βIII-tubulin, GFAP, MAP2b, and ACHE. In agreement with previous studies, un-transfected cells and cells transfected with Pax6 did not expressed genes specific for neuronal lineages (Table 25). On the other hand, following transfection with Msi1, levels of nestin and ACHE were increased to 4-fold and 8-fold, respectively, and this expression was maintained over the 12-day period. Also levels of GFAP mRNA was enhanced time dependently by approximately 14 times. Likewise, the same pattern was observed in Ngn2 transfected cells. While expression of βIII-tubulin and MAP2b were modestly increased following transfection with one neurogenic transcription factors the regulation of gene expression after transfecting the cells with two neurogenic factors, Msi1 or Ngn2 with Pax6, did not further increase the expression of neuronal genes. Expression of these genes was enhanced when the cells were transfected with Msi1 and Ngn2, with βIII-tubulin enhanced to almost 6-fold at day 12.

TABLE 25

RT-PCR analysis of relative expression of neuronal precursor cell markers such as nestin, βIII-tubulin, MAP2b, ACHE, and GFAP after transfection of fibroblast cells with pCMV6-XL5-Msi1, pCMV6-XL4-Ngn2, pCMV6-XL5-Pax6, and pCMV6-XL5-MBD2. After 24 h following transfection, CDM I Medium was changed and cells were cultured in proliferation medium (NPBM, Lonza) supplemented with EGF (20 ng/ml. Peprotech) and bFGF (20 ng/ml, Peprotech) for one week. Differentiation was induced by changing the medium to NbActive (BrainBits ™) supplemented with NT-3 (20 ng/ml), bFGF (20 ng/ml), ATRA (5 μM) and Forskolin (10 μM). Cells were incubated at 37° C., 5% CO_2, 5% O_2 for 12 days. Relative expression of Msi1, Ngn2, Pax6, nestin, βIII-tubulin, ACHE, MAP2b and GFAP in NSLCs and NLCs was increased after transfection with both transcription factors Ngn2 and Msi1 with MBD2 as the DNA demethylator.

	COL5A2		FBN2		NES		MAP2		TUBB3		SOX2		ACHE		GFAP	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1, +CytoB, Control	1.00	0.07	1.00	0.01	1.00	0.04	1.00	0.05	1.00	0.05	1.00	0.05	1.00	0.10	1.00	0.11
#2, −CytoB, Control	1.00	0.03	1.00	0.08	1.00	0.00	1.00	0.09	1.00	0.09	1.15	0.80	1.01	0.18	1.00	0.01
#3, +CytoB, Msi1, GAD45b	0.85	0.04	0.75	0.02	0.60	0.01	0.29	0.01	0.44	0.00	22.39	5.26	0.81	0.19	10.14	0.15
#4, −CytoB, Msi1, GAD45b	0.87	0.03	1.81	0.09	1.84	0.04	2.31	0.00	2.09	0.03	20.28	5.33	1.99	0.74	6.03	0.05

TABLE 25-continued

RT-PCR analysis of relative expression of neuronal precursor cell markers such as nestin, βIII-tubulin, MAP2b, ACHE, and GFAP after transfection of fibroblast cells with pCMV6-XL5-Msi1, pCMV6-XL4-Ngn2, pCMV6-XL5-Pax6, and pCMV6-XL5-MBD2. After 24 h following transfection, CDM I Medium was changed and cells were cultured in proliferation medium (NPBM, Lonza) supplemented with EGF (20 ng/ml. Peprotech) and bFGF (20 ng/ml, Peprotech) for one week. Differentiation was induced by changing the medium to NbActive (BrainBits ™) supplemented with NT-3 (20 ng/ml), bFGF (20 ng/ml), ATRA (5 μM) and Forskolin (10 μM). Cells were incubated at 37° C., 5% CO_2, 5% O_2 for 12 days.
Relative expression of Msi1, Ngn2, Pax6, nestin, βIII-tubulin, ACHE, MAP2b and GFAP in NSLCs and NLCs was increased after transfection with both transcription factors Ngn2 and Msi1 with MBD2 as the DNA demethylator.

	COL5A2		FBN2		NES		MAP2		TUBB3		SOX2		ACHE		GFAP	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#5, +CytoB, Ngn2, GAD45b	0.84	0.04	0.77	0.03	0.44	0.00	0.24	0.00	0.36	0.01	470.84	13.43	0.63	0.05	103.22	0.80
#6, −CytoB, Ngn2, GAD45b	0.75	0.07	1.97	0.02	1.83	0.00	4.40	0.16	2.02	0.10	789.33	60.35	1.70	0.13	110.48	4.90
#7, +CytoB, Pax6, GAD45b	0.74	0.12	1.08	0.00	0.89	0.01	0.51	0.00	0.63	0.04	1.64	0.98	0.86	0.12	2.49	0.21
#8, −CytoB, Pax6, GAD45b	0.66	0.04	2.41	0.09	2.70	0.03	4.96	0.30	3.48	0.07	0.46	0.33	2.97	1.04	0.43	0.09
#9, +CytoB, Msi1, Ngn2, GAD45b	0.14	0.01	0.28	0.01	1.30	0.03	4.07	0.11	0.84	0.00	54768.27	6709.56	0.81	0.24	3391.96	64.63
#10, −CytoB, Msi1, Ngn2 GAD45b	0.12	0.00	0.73	0.03	5.28	0.21	50.84	1.23	4.93	0.28	17400.66	822.88	3.58	0.10	1255.76	5.27
#11, +CytoB, Msi1, Ngn2 MBD2	0.10	0.00	0.26	0.01	1.11	0.01	3.69	0.09	0.76	0.00	55588.41	1331.20	0.55	0.14	2849.96	261.51
#12, −CytoB, Msi1, Ngn2 MBD2	0.44	0.01	1.47	0.06	5.49	0.14	47.30	0.11	5.50	0.31	14587.46	789.19	3.90	0.13	1424.04	39.29
#13, +CytoB, GAD45b	1.11	0.04	1.09	0.06	0.92	0.08	0.68	0.01	0.82	0.03	63.93	2.81	1.19	0.17	17.43	1.86
#14, −CytoB, GAD45b	0.94	0.01	2.22	0.00	2.82	0.02	6.49	0.30	4.01	0.05	6.12	0.61	2.34	0.17	1.42	0.10
#15, +CytoB, MBD2	0.83	0.00	0.83	0.05	0.36	0.01	0.16	0.01	0.36	0.00	3.42	3.74	0.63	0.37	2.18	0.12
#16, −CytoB, MBD2	0.68	0.02	1.55	0.04	1.57	0.05	1.47	0.01	2.00	0.00	0.52	0.29	1.45	0.15	0.55	0.04
#17, +CytoB, Msi1, Ngn2	1.10	0.01	1.16	0.03	1.37	0.01	1.12	0.06	0.86	0.06	5.59	1.48	1.07	0.27	1.70	0.46
#18, −CytoB, Msi1, Ngn2	0.93	0.04	2.52	0.10	3.48	0.01	9.01	0.02	4.55	0.18	1.78	1.46	3.83	0.42	0.59	0.01
#19, +CytoB, Msi1, MBD2	0.20	0.03	0.36	0.01	1.25	0.05	6.68	0.31	0.72	0.02	66592.29	3481.89	2.57	0.03	4450.08	131.85
#20, −CytoB, Msi1, MBD2	0.12	0.00	0.64	0.03	4.70	0.22	77.51	0.11	4.12	0.11	19128.03	1542.00	8.14	0.13	999.22	24.75

TABLE 25-continued

RT-PCR analysis of relative expression of neuronal precursor cell markers such as nestin, βIII-tubulin, MAP2b, ACHE, and GFAP after transfection of fibroblast cells with pCMV6-XL5-Msi1, pCMV6-XL4-Ngn2, pCMV6-XL5-Pax6, and pCMV6-XL5-MBD2.
After 24 h following transfection, CDM I Medium was changed and cells were cultured in proliferation medium (NPBM, Lonza) supplemented with EGF (20 ng/ml. Peprotech) and bFGF (20 ng/ml, Peprotech) for one week. Differentiation was induced by changing the medium to NbActive (BrainBits ™) supplemented with NT-3 (20 ng/ml), bFGF (20 ng/ml), ATRA (5 μM) and Forskolin (10 μM). Cells were incubated at 37° C., 5% CO_2, 5% O_2 for 12 days.
Relative expression of Msi1, Ngn2, Pax6, nestin, βIII-tubulin, ACHE, MAP2b and GFAP in NSLCs and NLCs was increased after transfection with both transcription factors Ngn2 and Msi1 with MBD2 as the DNA demethylator.

	COL5A2		FBN2		NES		MAP2		TUBB3		SOX2		ACHE		GFAP	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#21, +CytoB, Ngn2, MBD2	0.17	0.01	0.28	0.00	1.16	0.04	5.73	0.06	0.62	0.00	67945.51	3000.74	2.15	0.04	4736.83	11.92
#22, −CytoB, Ngn2, MBD2	0.17	0.00	0.78	0.03	4.32	0.08	68.89	5.26	4.01	0.04	16570.91	92.96	7.04	0.53	1427.13	13.19
#23, +CytoB, Msi1	0.71	0.05	0.79	0.06	0.87	0.01	0.63	0.06	0.67	0.04	2.86	0.70	1.08	0.08	2.08	0.11
#24, −CytoB, Msi1	0.66	0.04	1.92	0.17	2.03	0.02	2.77	0.02	2.68	0.02	0.32	0.12	1.85	0.65	0.58	0.04

Same pattern of gene expression was observed when transfecting the cells with three transcription factors (Msi1, Ngn2, and Pax6), but the expression was less pronounced than in cells transfecting with just Msi1 and Ngn2. In terms of immunohistochemical analysis after the 12 days of the transfection, cells displayed neuronal markers after transfection with Msi 1 or Ngn2, as indicated by the expression of nestin and MAP2b. Cells transfected with pCMV-XL-PAx6 did not stain for Nestin and MAP2b.

This study shows that transfecting cells within CDM with only one neurogenic factor (Msi1 or Ngn2) induces morphological changes and expression of one or more markers of neural stem cells and neuronal cells. Since the reprogrammed cells expressed a key neurogenic factor, a neuronal precursor marker, and a mature neuronal marker at low percentage (10%), this suggests that cells within the CDM were transformed to NSLCs and then started to differentiated through the various phases of the neuronal determination and differentiation program induced in neural stem cells.

Example XI

Gene Expression Analysis of Reprogrammed Cells within CDM

This study was designed to test the effect of transfecting cells with Msi1 and Ngn2 in the presence of MBD2 in the reprogramming process. Cells were transfected after two days of pre-treatment with cytocahiasin B with the DNA expression vectors using Lipofectamine reagent as described in Example X. 15 μg of eukaryotic DNA expression vectors pCMV6-XL5-Musashi or pCMV6-XL4-Ngn2, and pCMV6-XL5-MBD2 (Origene), were used to co-transfect cells. After 24 hours, the media was changed to CDM: Neural Progenitor Maintenance Medium (1:1) supplemented with Noggin (50 ng/ml), EGF (20 ng/ml), and bFGF (20 ng/ml), Medium was changed every day by increasing the percentage of NPBM and decreasing CDM medium. Cells were cultured for 6 days at 37° C., 5% $CO_2$and 5% O_2. After one week, differentiation was initiated by gradually supplementing the NPBM Medium with NT-3 (20 ng/ml, Peprotech), all-trans-retinoic acid (ATRA, 5 μM, Spectrum), BDNF (20 ng/ml, Peprotech), and bFGF (40 ng/ml, Peprotech). Samples were collected at the end of the study (day 14) and data were analyzed by gene array to identify genes that were reproducibly found to be specific for neuronal lineages.

Gene Expression Analysis

Gene expression analysis on 8 samples was performed as previously described in Example I with the customized Neuronal Markers 2 TLDA In order to identify the expression of genes related to neural stem cells, neuronal cells and glial cells, and growth factors expressed by the cells after transfection. The expression of oligodendrocyte genes, such as NKx2.2, olig2, and MAG was increased by Msi1 and Ngn2; however, the increased was more pronounced by Msi1 as compared to Ngn2 (Table 26). Two markers for astrocytes (GFAP and AQP4) were highly expressed after transfection with Msi1 and Ngn2 in the presence of the DNA demethylator MBD2. Interestingly, several markers of early neuronal cells were enhanced; 12 days after transfection, TDLA data revealed increases in specific markers for interneurons, such as somatostatin and calbindin1. Doublecortin (DCX), which is expressed by migrating immature cells during development, and acetylcholine (ACHE), an early marker of neuronal cells, were highly expressed in reprogrammed cells (Table 26). Transfection with Msi1 or Ngn2 increased the expression of dihydropyrimidinase-like 3 (DPYSL3), an early marker of newborn neurons to five-fold with Msi1 and seven-fold with Ngn2. Expression of microtubule-associated protein 2 (MAP2), an essential marker for development and maintenance of early neuronal morphology, and neuronal cell adhesion molecule (NCAM) were highly expressed with Msi1 and Ngn2. The expression of enolase-2, a marker of mature neurons, was 20-fold enhanced by Msi1 and Ngn2. Member of the NeuroD family NeuroD1 was highly expressed after transfection with Msi1 to 84.22 fold and to 34.27 by Ngn2. Gene expression of growth factors such as IGF-1, IGF-2, NPY and CSF-3 was enhanced following transfection with Msi1 or Ngn2. The expression of VEGF and GDNF genes were increased to almost five-fold and seven-fold by Msi1 and Ngn2, respectively. However in transfected cells, the expression of BDNF, EGF, and bFGF were not activated and even down-regulated as compared to untransfected cells. The expression of growth associated protein (GAP-43), a growth- and regeneration-associated marker of neurite extension, and expression of netrin, implicated in neuronal development and guidance, were highly expressed in transfected cells

(Table 26). Expression of receptors for growth and neurotrophic factors was increased, such as type III receptor tyrosine kinase, Neurotrophic tyrosine kinase receptor, and neurotrophic tyrosine kinase. The fibroblast-specific markers vimentin and fibronectin were down-regulated in the reprogrammed cells.

Transfection of HFF with only Msi1 and Ngn2 in the presence of MBD2 increased the expression of glial cells and neuronal cells markers.

TABLE 26

Gene array of CDM transfected with pMsi1 and pNgn2 following the pre-treatment with cytochalasin B (10 μg/ml), VPA (4 mM) and 5-Azacytidine (5 μM). Transfected cells were cultured in differentiation medium (NbActive, BrainBits ™) supplemented by ATRA (5 μM), bFGF (40 ng/ml) and BDNF (20 ng/ml).

Symbol	Common name and description	Company Gene ID	Relative expression Msi1	Relative expression Ngn2
Astrocytes and oligodendrocytes markers				
NKx2-2	Markers for oligodendrocyte progenitors	NM_002509.2	1.72	10.19
OLIG2	Oligodendrocyte lineage transcription factor 2	NM_005806.2	1.72	1.52
MBP	Myelin-basic protein	NM-001025090.1	1.72	1.52
GFAP	Glial fibrillary acidic protein	NM_002055.4	6.04	2.41
AQP4	Aquaporin 4	NM_001650.4	1.72	1.52
DIO2	Deiodinase iodothyronine type II	NM_013989.3	8.29	10.61
NC markers				
SST	Somatostatin, specific marker for interneurons	NM_001048.3	very high	very high
CALB1	Calbindin 1, interneuron marker	NM_004929.2	1.72	1.52
Tubulin1A	Are necessary for axonal growth	NM_006009.2	0.63	0.76
NES	Precursor neurons (nestin)	NM_006617.1	2.42	2.86
DCX	An early neuronal marker (Doublecortin)	NM_178151.1	1.72	1.52
ACHE	Acetylcholinesterase, marker of early neuronal development	NM_015831.2	10.68	20.37
ENO2	A marker for neurons cells, enolase	NM_001975.2	0.55	0.54
NEUROD1	Neural marker; expression gradually increased from neural precursor to fully differentiated neuron	NM_002500.2	1.72	1.50
DPYSL3	Dihydropyrimidinase-like 3, marker of immature neurons	NM_001387.2	0.62	0.71
MAP2	Microtubule-associated protein 2, essential for development of early neuronal morphology and maintenance of adult neuronal morphology	NM_002374.3	1.99	1.70
NCAM	Neural cell adhesion molecule 1	NM_18135.3	3.11	5.72
CEND1	Cell cycle exit & neuronal differentiation, early marker of proliferating precursor cells that will differentiate to neurons	NM_016564.3	6.68	8.28
Neuroregeneration and survival genes				
FGF2	Fibroblast growth factor	NM_002006.4	1.19	1.26
EGF	Epidermal growth factor,	Hs00153181_m1	28.37	52.13
IGF-1	Insulin growth factor-1,	NM_000618.2	0.82	1.03
IGF-2	Insulin growth factor-2	NM_0000612.3	0.99	1.21
CSF3	Granulocyte colony-stimulating factor	NM_2219.1	very high	very high
BDNF	Brain derived growth factor, neurogenesis	NM-199231.1	8.54	7.84
GDNF	Glial derived neurotrophic factor	NM-000614.2	0.63	0.91
CNTF	Ciliary neurotrophic factor	NM_001025366.1	3.80	14.92
VEGF	Vascular endothelial growth factor	NM_130850.1	6.28	7.22
BMP-4	Bone morphogenetic protein 4	NM_002253.1	1.17	1.34
KDR	Type III receptor tyrosine kinase)	NM_006180.3	113.85	43.87
NTRK2	Neurotrophic tyrosine kinase recepto (TrkB)	NM_000905.2	0.02	0.02
NPY	Neuropeptide Y	NM_009905.2	33.39	1.52
NTF-5	Neurotrophin 5	NM_006179.3	4.43	5.93
PIK3CG	phosphoinositide-3-kinase,	NM_002649.2	1.70	1.50
STAT3	Signal transduction transcription 3	NM_213662.1	3.15	2.24
Gap43	Growth associated protein 43	NM_002045.2	1.82	2.98
NTN1	Netrin1, implicated in neuronal development and guidance	NM_004822.2	0.50	0.29
NTRk2	Neurotrophic tyrosine kinase, receptor, type 2	NM_006180.3	0.02	0.02
L1CAM	L1 cell adhesion molecule, associated with regenerating axons	NM_024003.1	0.08	0.11
LIMK1	LIM domain kinase 1	NM_002314.2	2.88	2.96

TABLE 26-continued

Gene array of CDM transfected with pMsi1 and pNgn2 following the pre-treatment with cytochalasin B (10 μg/ml), VPA (4 mM) and 5-Azacytidine (5 μM). Transfected cells were cultured in differentiation medium (NbActive, BrainBits ™) supplemented by ATRA (5 μM), bFGF (40 ng/ml) and BDNF (20 ng/ml).

Symbol	Common name and description	Company Gene ID	Relative expression Msi1	Relative expression Ngn2
Vimentin	Radial glia and fibroblast marker	NM-003380.2	0.21	0.20
Fibronectin	fibronectin is a marker for fibroblasts	NM_212474.1	0.15	0.14

Example XII

Reprogramming of Cells within CUM by Lipofectamine and Nucleofection

This study was designed to improve transfection of CDM by combining lipofectamine and nucleofection and using two vectors pCMV6-XL5-Msi1 and pCMV6-XL4-Ngn2 individually or in combination together with pCMV-XL5-MBD2. Cells within Day 4 CDM were lipotransfected for 6 hours with Msi1/MBD2, Ngn2/MBD2 or Msi/Ngn2/MBD2 after 2 days of pre-treatment with or without cytochalasin B. In parallel, transfection was performed on fresh HFFs after the 6 hours using Nucleofection as described in Example II, and transferred on top of the CDM when the lipofectamine media was changed to fresh CDM medium. After 24 hours, the medium was changed to Neural Progenitor Basal Medium (NPBM, Lonza) with the presence of Noggin (50 ng/ml, Peprotech), recombinant hFGF (20 ng/ml, Peprotech), and recombinant hEGF (20 ng/ml, Peprotech). Differentiation was induced at day 7, by adding NSA-A differentiation medium (StemCell Technologies) for 21 days.

Gene Expression Analysis

Samples were collected at 8, 15, and 21 days to evaluate the nature of newly formed cells by analyzing the expression of several neuronal marker genes using RT-PCR according to the methods previously described in Example I. As shown in Table 27, cells transfected with one neurogenic transcription factor (Msi1 or Ngn2) express high levels of nestin and βIII-tubulin at day 8. The same pattern of expression was observed at day 15 and 21, while the expression was slightly decreased in the absence of cytochalasin B in cells transfected with Ngn2. The expression of all genes, except the mature neuronal marker MAP2b, were remarkably increased in cells transfected with both neurogenic transcription factors. The upregulation of these genes was slightly reduced in the absence of cytochalasin B, indicating its role in enhancing reprogramming.

TABLE 27

RT-PCR analysis of relative expression of neuronal stem cell markers such as nestin, Sox2, and GFAP after transfection of fibroblast cells within the CDM with different combinations with or without the co-treatment with cytochalasin B. Relative expression of Sox2, nestin, and GFAP in NSLCs was increased after transfection with both transcription factors Ngn2 and Msi1 with MBD2 as the DNA demethylator.

	MSI1		NGN2		TUBB3		GFAP		NES		MAP2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1 Day 8 CDM − CytoB Control	1.11	0.21	1.33	0.20	1.10	0.02	0.91	0.02	1.18	0.09	0.91	0.02
#2 Day 8 CDM − CytoB Control	1.11	0.17	0.65	0.08	0.92	0.06	0.91	0.11	0.82	0.01	0.91	0.11
#3 Day 8 CDM − CytoB Control	0.83	0.01	0.71	0.86	0.99	0.04	1.21	0.00	1.03	0.00	1.21	0.00
#4 Day 8 CDM + CytoB Control	7.42	0.35	1.52	0.53	1.32	0.16	0.44	0.06	1.04	0.02	0.44	0.06
#5 Day 8 CDM + CytoB Control	7.01	0.42	2.14	0.58	1.23	0.07	0.62	0.05	1.02	0.06	0.62	0.05
#6 Day 8 CDM + CytoB Control	9.15	0.48	0.76	0.08	0.40	0.05	0.59	0.14	0.34	0.16	0.59	0.14
#7 Day 15 CDM − CytoB Control	1.45	0.07	1.53	0.33	1.32	0.01	0.90	0.07	1.31	0.03	0.90	0.07
#8 Day 15 CDM − CytoB Control	0.79	0.02	2.01	1.49	0.91	0.03	1.14	0.16	0.91	0.01	1.14	0.16
#9 Day 15 CDM − CytoB Control	0.87	0.04	0.64	0.72	0.84	0.08	0.98	0.15	0.84	0.01	0.98	0.15
#10 Day 15 CDM + CytoB Control	1.27	0.14	0.99	0.66	1.70	0.21	0.36	0.02	1.08	0.08	0.36	0.02
#11 Day 15 CDM + CytoB Control	1.39	0.04	0.97	0.65	2.65	0.38	0.44	0.06	1.97	0.30	0.44	0.06
#12 Day 15 CDM + CytoB Control	1.09	0.21	0.49	0.46	1.32	0.14	0.47	0.15	2.45	0.15	0.47	0.15
#13 Day 21 CDM − CytoB Control	1.21	0.00	1.06	0.06	1.10	0.01	0.86	0.16	1.07	0.01	0.86	0.16
#14 Day 21 CDM − CytoB Control	0.97	0.09	2.16	0.77	0.96	0.01	1.11	0.10	0.94	0.01	1.11	0.10
#15 Day 21 CDM − CytoB Control	0.86	0.02	1.01	1.27	0.94	0.00	1.08	0.26	0.99	0.04	1.08	0.26
#16 Day 21 CDM + CytoB Control	1.41	0.21	1.29	1.64	2.46	0.07	0.88	0.22	1.58	0.05	0.88	0.22
#17 Day 21 CDM + CytoB Control	2.24	0.00	0.35	0.01	2.23	0.03	0.55	0.16	1.57	0.02	0.55	0.16
#18 Day 21 CDM + CytoB Control	2.18	0.14	0.77	0.06	2.29	0.12	0.54	0.04	1.47	0.04	0.54	0.04
#19 Day 8 CDM − CytoB Msi1/MBD2	694.16	18.10	0.51	0.05	1.46	0.04	2.18	0.13	1.02	0.03	2.18	0.13

TABLE 27-continued

RT-PCR analysis of relative expression of neuronal stem cell markers such as nestin, Sox2, and GFAP after transfection of fibroblast cells within the CDM with different combinations with or without the co-treatment with cytochalasin B. Relative expression of Sox2, nestin, and GFAP in NSLCs was increased after transfection with both transcription factors Ngn2 and Msi1 with MBD2 as the DNA demethylator.

	MSI1		NGN2		TUBB3		GFAP		NES		MAP2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#20 Day 8 CDM − CytoB Ngn2/MBD2	2.38	0.29	4106.88	48.57	0.46	0.02	1.88	0.14	0.99	0.02	1.88	0.14
#21 Day 8 CDM − CytoB Msi1/Ngn2/MBD2	365.04	6.71	2702.81	55.69	4.44	0.02	2.95	0.38	5.11	0.05	2.95	0.38
#22 Day 8 CDM + CytoB Msi1/MBD2	1262.00	63.21	0.75	0.91	0.54	0.03	2.48	0.11	1.16	0.05	2.48	0.11
#23 Day 8 CDM + CytoB Ngn2/MBD2	2.34	0.20	10963.51	19.89	0.53	0.00	2.27	0.26	1.00	0.06	2.27	0.26
#24 Day 8 CDM + CytoB Msi1/Ngn2/MBD2	869.15	65.33	6401.28	87.12	4.58	0.01	3.65	0.13	3.15	0.00	3.65	0.13
#25 Day 15 CDM − CytoB Msi1/MBD2	41.07	1.74	2.58	0.36	1.43	0.05	0.58	0.06	1.34	0.07	0.58	0.06
#26 Day 15 CDM − CytoB Ngn2/MBD2	0.73	0.02	2192.64	15.74	0.95	0.08	1.01	0.09	0.99	0.03	1.01	0.09
#27 Day 15 CDM − CytoB Msi1/Ngn2/MBD2	45.59	2.33	3318.42	51.51	5.32	0.08	3.80	0.01	4.32	0.01	4.80	0.01
#28 Day 15 CDM + CytoB Msi1/MBD2	106.34	4.43	4.90	1.70	1.47	0.01	0.57	0.10	1.19	0.03	0.57	0.10
#29 Day 15 CDM + CytoB Ngn2/MBD2	1.09	0.11	6715.95	505.86	1.30	0.05	0.70	0.17	1.18	0.07	0.70	0.17
#30 Day 15 CDM + CytoB Msi1/Ngn2/MBD2	46.77	0.76	2816.33	90.83	5.76	0.02	4.52	0.09	3.60	0.03	5.52	0.09
#31 Day 21 CDM − CytoB Msi1/MBD2	22.94	1.09	10.09	2.72	1.08	0.07	0.58	0.08	1.17	0.02	0.58	0.08
#32 Day 21 CDM − CytoB Ngn2/MBD2	0.78	0.02	4450.56	255.75	1.00	0.03	0.75	0.21	1.09	0.03	0.75	0.21
#33 Day 21 CDM − CytoB Msi1/Ngn2/MBD2	24.02	0.86	2509.95	64.00	5.18	0.05	4.74	0.16	4.37	0.06	3.74	0.16
#34 Day 21 CDM + CytoB Msi1/MBD2	54.17	1.41	8.31	3.32	1.42	0.05	0.70	0.22	1.71	0.02	0.70	0.22
#35 Day 21 CDM + CytoB Ngn2/MBD2	1.19	0.15	1180.19	27.29	1.21	0.06	1.03	0.34	1.31	0.04	1.03	0.34
#36 Day 21 CDM + CytoB Msi1/Ngn2/MBD2	81.66	1.34	7789.96	345.72	5.24	0.05	5.84	0.10	4.37	0.05	5.84	0.10

Immunohistochemical Analysis

Samples were collected at 4, 8, 14, and 21 days to evaluate the nature of any reprogrammed cells by analyzing the expression of several neuronal markers using immunohistochemical analysis according to the methods previously described in Example I. The immunhistochemical analysis at various time points revealed that within the first 8 days the expression of nestin was induced in a large proportion of cells and decreased time-dependently after inducing the differentiation.

This study indicates that upon transfecting the cells with one or two neurogenic genes in the presence of cytochalasin B and MBD2, reprogrammed cells were stable in culture, responded to environmental changes (proliferation vs differentiation), and expressed neuronal markers for at least 24 days in culture.

Example XIII

Telomerase Activity of NSLCs

Telomerase is active in neural precursor cells and suggest that its regulation is an important parameter for cellular proliferation to occur in the mammalian brain (Caporaso G L et, 2003). This study was performed to evaluate telomerase activity in cell extracts of adherent NSLCs (NSLCs cultured on laminin-coated plates) as well as NSLCs in floating neurospheres (NSLCs cultured in plates with a low-bind surface) at early (P7) and late passage (P27). The telomerase activity of the 4 samples was measured by the PCR-based telomere repeat amplification protocol (TRAP) using the TRAPeze® Telomerase Detection Kit (Chemicon). Briefly, the cells were grown in 24-well plates, washed in PBS, and homogenized for 30 min on ice in buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS and 10% Glycerol (1× CHAPS Lysis Buffer, provided in kit) and RNase Inhibitor. The samples were spun down and the protein concentration of the supernatant was determined using the BCA Assay. 900 ng of protein from each cell extract was added directly to the TRAP reaction mixture containing TRAP reaction buffer, dNTPs, template substrate (TS) primer, TRAP primer mix and Taq polymerase. The reaction mixtures were incubated at 30° C. for 30 minutes for template synthesis, followed by a PCR procedure (95° C./15 min for initial denaturation, 94° C./30 sec, 59° C./30 sec, 72° C./1 min for 32 cycles) for amplification of the extended telomerase products. To detect telomerase activity, polyacrylamide gel electrophoresis (PAGE) was performed for the reaction products on a 10% non-denaturing TBE gel. After electrophoresis, the gel was stained with SYBR® Green I Nucleic Acid Gel Stain for 30 minutes, followed by image capture using a Gel-Documentation System (Alpha Innotech).

All 4 samples were telomerase positive (as indicated by the TRAP product ladder). As expected, the Heat-treated control (ΔH) showed no Telomerase activity (Negative Control). A 36 bp internal control band (S-IC) is used to monitor PCR amplification (to distinguish false-negative results). This S-IC band was observed for all samples except for the test samples. This may have been due to the excessively high telomerase activity in the test samples; amplification of the TRAP products and the S-IC control band are semi-competitive. All controls gave expected results (No TRAP products for CHAPS ctrl, and TRAP ladder of products for the positive control cells and the TSR8 control).

Example XIV

Tumor Formation Assay

Malignantly transformed cells show reduced requirements for extracellular growth promoting factors, are not restricted by cell-cell contact, and are often immortal. Anchorage-independent growth and proliferation is one of the hallmarks of malignant transformation, which is considered the most accurate and stringent in vitro assay for detecting malignant transformation of cells.

Adherent and neurosphere NSLCs at early and late passage (P7 and P25), as well as normal human neuroprogenitor cells (hNPCs), were investigated for the anchorage-independent growth. HFFs were used as a negative control and cervical carcinoma HeLa cells were used as a positive control. Cells were sedimented by centrifugation at 150×g for 3 min at room temperature (RT). The assay was performed using the CytoSelect™ 96-well cell transformation assay (CellBiolabs). The base agar layer (1.2%) was dissolved in 2×DMEM/20% PBS solution and 50 μl of the agar solution was added to the plate and incubated for 30 min at 4° C. to solidify. Prior to adding the cell agar layer, the plate was allowed to warm up for 15 minutes at 37° C. The cells were resuspended at different density (20.000 and 5000 cells/well), except the hNPCs were resuspended only at 5000 cells/well due to a lack of enough cells. The cells were mixed with the 1.2% agar solution, 2×DMEM/20% PBS, and cell suspension (1:1:1), and 75 μl of the mixture was transferred to wells already containing the solidified base agar layer, and was then placed in 4° C. for 15 minutes to allow the cell agar layer to solidify. 100 μl of proliferation medium (StemCell Technologies) was added and the plate was incubated for 8 days at 37° C. and 5% CO_2 before being solubilized, lysed and detected by the CyQuant™ GR dye in a fluorescence plate reader. The fluorescence measurement was performed using the Flexstation™ (Molecular Devices) with a 485/538 nm filter.

TABLE 28

Fluorescence measurement (Relative Fluorescence Unit, RFU) indicate that under the same conditions only carcinoma HeLa cells grow as an anchorage-independent colony, while both hNPCs and NSLCs (adherent and floating neurospheres) were negative for tumor growth in the standard agar plate tumor formation assay (CytoSelect ™ cell transformation kit, Cell Biolabs Inc.).

	Cell types			
Cell density	Hela	HFF	NSLCs	HNPCs
20.000	60.05 ± 8.70	14.82 ± 1.57	19.22 ± 1.85	19.00 ± 2.71
10.000	39.03 ± 3.97	13.73 ± 1.05	14.99 ± 1.12	21.61 ± 9.95
5000	24.70 ± 3.89	11.65 ± 0.57	12.29 ± 0.79	12.45 ± 0.73

As shown in Table 28, fluorescence measurement indicated that under the same conditions only carcinoma HeLa cells significantly grew and proliferated as anchorage-independent colonies, while both hNPCs and NSLCs (adherent and floating neurospheres) were negative for tumor growth (same value as HFFs (negative control) for 5,000 and 10,000 cells) in the standard agar plate tumor formation assay by visual observation of cells by light microscopic observation using bright field at 10× confirm Fluorescence measurement. Thus the transient transfection method and genes used allows the reprogramming of cells without the neoplastic transformation that generally occurs with stable transfection or certain genes via a series of genetic and epigenetic alterations that yield a cell population that is capable of proliferating independently of both external and internal signals that normally restrain growth.

Example XVI

No Genomic Integration of Plasmid DNA in NSLCs from Transient Transfection

The DNA plasmid Msi1/Ngn2 (designed and constructed in house) was used in transient transfection for generation of NSLCs along with MBD2 (for sample 1), or 5-Aza and VPA (for sample 2). Two weeks after transfection, Southern blot was performed to test for possible genomic integration of the plasmid DNA. 3 μg of genomic DNA extracted from the NSLC samples, as well as from HFF (a human fibroblast cell line) used as a negative control, was digested with several restriction enzymes including BglII, PstI and StuI, subjected to electrophoresis on a 1% agarose gel and transferred to a positively charged nylon membrane (Roche). The membrane was hybridized in the DIG Easy Hyb™ buffer (Roche) at 42° C. overnight with a 1.2 kb Dig-labeled PCR probe amplified from the plasmid DNA using a set of primers. The membrane was washed twice at room temperature with 2×SSC, 0.1% SDS for 5 min per wash, twice with 0.5×SSC, 0.1% SDS at 65° C. for 15 min per wash. Hybridization signals of the membrane were detected using the CDP-Star™ substrate (Roche). The membrane was exposed to an X-ray film for analysis. The signals were stripped from the membrane using stripping buffer (0.2 M NaOH, 0.1% SDS). The membrane was re-hybridized with a 0.9 kb Dig-labeled PCR probe amplified from the plasmid DNA using a set of primers.

The Southern blot analysis with the 1.2 kb Dig-labeled PCR probe revealed distinct signals in the positive control samples where the Msi1/Ngn2 plasmid DNA was spiked into HFF genomic DNA for the equivalence of 1, 10 or 100 integrations per genome. There were a few weak and identical bands that appeared in the restriction enzyme digested genomic DNA from HFF, NSLC samples #1 and #2, suggesting that there is no plasmid DNA integration in the genomic DNA of NSLCs. These bands may represent the endogenous Ngn2 gene since the 1.2 kb Dig-labeled PCR probe contains a small part of the Ngn2 gene. This data shows that no, or only a tiny number of, NSLCs had plasmid integration into the host genome after transient transfection, and that the transfected genes are only present in the cell for a short period of time (less than two weeks).

Example XVII

Neuroprotective Effect of Transplanted hNSLCs in

1) Animal Model of Multiple Sclerosis.

Multiple Sclerosis (MS) is an incurable inflammatory demyelinating disease of the central nervous system (CNS) (Frohman E M et al 2006). Therapies for MS rely on manipulation of the immune system, but with often modest effectiveness on reducing clinical episodes or permanent neurological disability, requiring frequent injections, and with sometimes-significant side effects (Langer-Gould A et al 2004). Experimental Allergic Encephalomyelitis (EAE) is an animal model of MS commonly used for studying disease mechanisms and testing potential therapies. EAE can be induced in a variety of species and strains of animals [mice, Rat, marmoset monkey, rhesus macaques] using various CNS antigens [Myelin Oligodendrocyte Glycoprotein (MOG), proteolipid protein (PLP) and myelin basic protein (MBP)].

After obtaining all appropriate animal approvals for the experiments, Female 7 to 8 weeks old C57BL/6 mice were purchased from Charles Rivers, and housed at MISPRO animal facility for one week before experimentation for adaption to the new environment. C57BL/6 mice were injected s.c. with 100 μg MOG 35-55 in CFA (Sheldon Biotechnology, McGill University) containing 5 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, inc), at 2 sites on the back. All mice received 200 ng pertussis toxin (List Biological Laboratories, Inc) i.p. on day 0 and 2, while clinical scores were calculated blindly daily during a 43 day period, according to the 0-5 scale as follows: 1, limp tail or waddling gait with tail tonicity; 2, waddling gait with limp tail (ataxia); 2.5, ataxia with partial limb paralysis; 3, full paralysis of 1 limb; 3.5, full paralysis of 1 limb with partial paralysis of second limb; 4, full paralysis of 2 limbs; 4.5, moribund; and 5, death.

Treatment of EAE Animal Model with and without the Cells:

hNSLC and hNPCs (1.5×10^6 cells in 200 μl PBS/each mouse) were given by single injection i.v. via the tail vein when the animals started to show symptoms of EAE (day 13 i.v). Both animals groups received cyclosporine (10 mg/kg/day) one day before the injection of cells and daily from the day of transplantation to avoid any rejection of the human cells. Sham-treated age-, sex-, and strain-matched mice, injected i.p. with PBS alone, were used as controls. All groups of animals were observed for 43 days. Animals were sacrificed at 43 days p.t., brains and spinal cord were harvested in 30% sucrose in PBS. Statistical analysis of the clinical scores revealed that the clinical signs of EAE were significantly attenuated in NSLC-injected animals as compared to control and hNPCs-injected animals. Cumulative scores was significantly reduced in the NSLC transplanted animals and the treatment has no effect on body weight.

2) Hemiplegic Animal Model (Unilateral Ablation of the Left Sensorimotor Cortex in Adult Rats).

After obtaining all appropriate animal approvals for the experiments, 8 rats per group (Sprague-Dawley, 250-300 g, Charles River) were anaesthetized using ketamine (Bimeda-MTC)/xylazine (50/10 mg/kg, Novopharm) and placed onto a stereotaxic frame. A midline cranial incision was performed with a sterile surgical scalpel blade, the cranial vault exposed and the bregma identified. The skull above the sensorimotor cortex was opened and the sensorimotor cortex area [0.5-4.0 mm caudal to bregma and 1.8-3.8 mm lateral to the midline (Paxinos and Watson 1986)] was carefully aspirated. After ablation, the treatments (Alginate, Alginate+hNPC, Alginate+NSLCs, RM_x+NSLCs, RM_x Only, Fibrin Gel, or Saline) were applied directly on the brain after ablation. The opening in the skull was then filled with Bone Wax. In case of a bleeding, small pieces of sterile homeostatic tissue were inserted into the lesion in order to stop the bleeding. The sutures were performed using Ethicon™ monofilament suture ½ circle needle shape. Surgeries were performed in sterile clean rooms, and topical antibiotics (Cicatrin®, GlaxoSmithKline) were applied to the exposed skull and scalp to limit local infection. Rats were immunosuppressed by daily injection i.p. of cyclosporine A (10 mg/kg/day) starting the day before the surgery until the end of the study period. The purpose of the cyclosporine A injection was to reduce the rat's immune reaction to the treatment. The immune-suppression was sustained until the end of the study to ensure that any potential failure of regeneration (if taken place) was not due to the immune reaction against the treatment. Functional scores were performed weekly, in all groups, sensorimotor impairment was evaluated based on the behavioural tests as described below.

Rotarod Test:

The rotarod speed was manually calibrated for the 10 and 20 RPM speed before all procedures. Animals were required to perch on the stationary rod for 30 sec to acclimate themselves to the environment. During this time, if any animal fell, it was placed back on the rod until it had achieved stationary capabilities for a period of 30 seconds. The animals were allowed 3 trials. The animals that were comfortable staying on the stationary rod for 30 sec were allowed to run with a constant speed of 10 and 20 RPM for 60 sec, and the number of falls were electronically recorded.

Beam Walking:

Beam walking measures hindlimb coordination by means of distance traveled across 100 cm beam (2.3 cm in diameter, 48 cm off the floor). Rats were systematically trained to walk along the elevated beam from start to finish with the aim of completing the task. A safe location, i.e, a flat box, is placed at the end of the beam so that the rat is motivated to complete the task.

Scale Used for Evaluation of Beam-Walking Performance

Scale	Performance characteristic
1	Animals fail to traverse the beam and do not place the hindlimb on the horizontal surface of the beam
2	Animals fail to traverse the beam, but place the hindlimb on the horizontal surface of the beam and maintain balance
3	Animals traverse the beam while dragging the hindlimb
4	Animals traverse the beam and place the hindlimb at least once during the traverse
5	Animals traverse the beam using the hindlimb to aid less than 50% of its steps on the beam
6	Animals traverse the beam using the left hindlimb to aid more than 50% of its steps on the beam
7	Animals traverse the beam with no more than two foot slips
8	Normal animals

Before the surgery, all the animals fell at least once from the rotarod, not because they had a walking or coordination problem, but because the speed was high. After the surgery (2 days), all the animals showed signs of significant walking and coordination problems leading to an increase in the number of falls from the rotarod. Three weeks after the surgery, the number of falls was clearly reduced for the animals receiving NSLCs as treatment compared to controls.

Animals passed the beam-walking test before the surgery without any difficulty. The rats crossed the 100 cm beam and got to the safe spot without falling off the beam. Two days after surgery, all groups completely failed to pass the test, and the animals were not able to stay in balance on the beam. One week after the surgery, all the animals showed some improvement in their walking capacity, but no significant difference was noticeable between the different treated groups. From week 4 until week 26, the animals treated with NSLCs as well as RM_x showed significant improvements in their walking capacity compared to the controls.

Example XVIII

Transfection of HFF by Various Combinations of Genes Using the Shuttle® Device and Treatment with Different Small Molecules for Reprogramming to Mesendoderm-Like Cells

HFF cells were cultured as described in CDM II medium as described in Example I with only modifying EGF (5 ng/ml) and FGF (10 ng/ml), and transfecting using the Nucleofector™® 96-well Shuttle® Device (Lanza) following the procedure described in Example IV. The cells were transfected with various combinations of cDNA clones as described in Table 29. After transfection, the cells were plated on 0.1% Gelatin-coated plates and incubated at 37° C., 5% CO_2, 5% O_2. Medium was changed every other day according to Table 30. Cells were analyzed at Day 4 by Quantitative Real-time PCR.

TABLE 29

Various combinations of plasmids with potential to transfect the cells towards mesendoderm lineage.

	Day −2 to Day 0	Plasmids transfected at Day 0[1]
1	Untreated	Oct4, FoxD3, MBD2
2		Oct4, T, MBD2
3		Oct4, Mixl1, MBD2
4		Oct4, Sox17, MBD2
5		FoxD3, T, MBD2
6		FoxD3, Mixl1, MBD2
7		FoxD3, Sox17, MBD2
8		T, Mixl1, MBD2
9		T, Sox17, MBD2
10		Mixl1, Sox17, MBD2
13	Pre-treated	Oct4, FoxD3
14	with	FoxD3, T
15	VPA & 5-Aza	FoxD3, Mixl1
16		FoxD3, Sox17
17		Oct4, FoxD3, T
18		Mixl1, Sox17, FoxA2
19		Oct4, FoxD3, T
20		Mixl1, Sox17, FoxA2

[1]where Oct4 = pCMV6-XL4-Oct4, FoxD3 = pCMV6-XL5-FoxD3, MBD2 = pCMV6-AC-MBD2, T = pCMV6-XL5-T, Mixl1 = pCMV6-XL5-MIXL1, Sox17 = pCMV6-XL4-SOX17, FoxA2 = pCMV6-XL5-FOXA2. All clones were purchased from Origene and prepared using the EndoFree Plasmid Maxi Kit (Qiagen).

TABLE 30

Medium composition from Day −2 to Day 10
Media Composition[2]

Day 0	Day 1	Day 2 to Day 3	Day 4 to Day 7	Day 8 to Day 10
CDM II (3:1 of DMEM:F12; GlutaMAX ™ 100x, Dexthamesone, 19.7 μg/ml, Glutathione (500 μg/ml, L-Ascorbic 75 mg/ml, Selenious acid 2.5 μg/ml, Insulin solution 10 mg/ml, T3 675 ng/ml, ethanolamine 500X, bFGF 2.5 ug/ml, and Egf (1.25 ug/ml) + Activin A + HSA	CDM II (50%) + IMDM/F12 (50%) + NEAA + ITS + HSA + bFGF + EGF + VPA + Activin A + CHIR99021	IMDM/F12 + NEAA + ITS + HSA + bFGF + EGF + VPA + Activin A + CHIR99021 + BMP4	IMDM/F12 + NEAA + ITS + HSA + bFGF + EGF + Activin A + CHIR99021 + BMP4	IMDM/F12 + NEAA + ITS + HSA + bFGF + EGF + BMP4

[2]Supplements added to media at the following concentrations: Activin A (Peprotech, 30 ng/ml), HSA (Baxter, 0.5%), NEAA (Gibco, 1X), ITS (Gibco, 1X), EGF (Peprotech, 5 ng/ml), bFGF (Peprotech, 10 ng/ml), CHIR99021 (Stemgent, 2 uM), VPA (Stemgent, 1 mM), 5-Aza (Sigma, 0.5 uM), BMP4 (Peprotech, 10 ng/ml)

Cells were collected on Day 4 by detaching with TrypLE™, followed by centrifugation at 80×g for 5 minutes. Supernatant was aspirated and the cell pellet was frozen at −86° C. until ready for RNA Isolation. RNA isolation and quantification was performed as previously described in Example I. cDNA was prepared and quantitative real-time PCR was performed as previously described in Example II, except the following Taqman™® Gene Expression Assays (Applied Biosystems) were used:

Gene	Taqman ™ ® Assay ID
GAPDH (housekeeper)	Hs99999905_m1
PPIA (housekeeper)	Hs99999904_m1
FOXA2	Hs00232764_m1
SOX17	Hs00751752_s1
Endogenous T	Hs00610073_g1
GSC	Hs00418279_m1
CXCR4	Hs00607978_s1
GATA4	Hs00171403_m1
CER1	Hs00193796_m1
CDH1 (E-cadherin)	Hs01023894_m1
p63	Hs00978340_m1
SOX2	SOX2_1078-ANY

TABLE 31

Relative Expression FoxA2, Sox17, and Cxcr4 after transfecting HFFs once with various gene combinations with potential to reprogram cells into mesoendoderm-like cells. The exact values are not significantly accurate due to low RNA yield, however a trend of increasing gene expression was detected for FoxA2, Sox17, and CXCR4.

	FOXA2		SOX17		CXCR4	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
Untreated HFF	1.00	0.04	1.00	0.04	1.00	0.04
Day 4 HFF untransf. (+G.F),	1.01	0.06	1.01	0.06	4.77	2.51
Day 4 HFF untransf. (−G.F),	1.38	0.11	1.38	0.11	1.38	0.11
Day 4 HFF Untransf. (+G.F.),	0.98	0.02	0.98	0.02	3.32	3.31
Day 4 HFF Untransf. (−G.F.),	4.12	4.07	1.28	0.06	1.28	0.06
Day 4 Oct4/FoxD3/MBD2	4.67	4.60	3.19	2.78	76.43	7.91
Day 4 Oct4/T/MBD2	3.91	3.55	4.33	2.36	15.18	2.52
Day 4 Oct4/Mixl1/MBD2	2.66	1.77	10.33	0.43	7.31	3.21
Day 4 Oct4/Sox17/MBD2	14.19	4.85	413533.31	127089.61	56.04	0.71
Day 4 FoxD3/T/MBD2	38.62	38.00	3.12	1.32	42.41	5.23
Day 4 FoxD3/Mixl1/MBD2	7.76	5.29	2.41	0.30	137.17	27.74
Day 4 FoxD3/Sox17/MBD2	26.02	1.95	50904.45	1523.33	131.03	17.53
Day 4 T/Mixl1/MBD2	3.67	3.26	5.64	4.15	14.04	2.89
Day 4 T/Sox17/MBD2	9.76	9.70	209797.21	24533.81	111.35	16.40
Day 4 Mixl1/Sox17/MBD2	3.60	3.10	237310.10	57448.60	36.76	1.07
Day 4 Oct4/FoxD3	13.87	0.16	13.87	0.16	35.44	14.57
Day 4 FoxD3/T	60.93	60.18	19.45	1.51	19.45	1.51
Day 4 FoxD3/Mixl1	21.20	2.31	28.96	8.66	62.31	55.82
Day 4 FoxD3/Sox17	96.88	3.60	54177.20	3313.15	44.57	41.51
Day 4 Oct4/FoxD3/T	25.99	18.15	12.27	1.26	21.17	11.33
Day 4 Mixl1/Sox17/FoxA2	1850864.68	98259.84	112641.65	15923.21	23.18	23.10
Day 4 Oct4/FoxD3/T (IMDM/F12)	9.52	5.61	1.52	0.02	35.74	4.36
Day 4 Mixl1/Sox17/FoxA2 (IMDM/F12)	486705.82	19101.53	57060.09	1262.81	13.44	2.36

TABLE 32

Expression of GATA4, CDH1 (E-cadherin), p63, and SOX2 relative to untreated HFF control 4 days after transfecting HFF cells with various gene combinations with potential to reprogram cells into mesoendoderm-like cells.

	GATA4		CDH1 (E-cadherin)		p63		SOX2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
Untreated HFF	1.00	0.04	1.00	0.04	1.00	0.04	1.00	0.04
Day 4 HFF untransf. (+G.F),	12.13	0.70	1.01	0.06	3.09	1.45	1.11	0.21
Day 4 HFF untransf. (−G.F),	4.48	0.85	1.38	0.11	3.11	2.54	1.38	0.11
Day 4 HFF Untransf. (+G.F.),	2.37	2.00	0.98	0.02	4.41	4.40	1.94	1.34
Day 4 HFF Untransf. (−G.F.),	6.12	3.33	1.28	0.06	13.23	7.43	1.28	0.06
Day 4 Oct4/FoxD3/MBD2	95.23	27.44	98.90	21.58	1.81	0.86	12.72	1.53
Day 4 Oct4/T/MBD2	33.66	10.30	1.42	0.02	2.05	0.87	2.62	1.67
Day 4 Oct4/Mixl1/MBD2	106.33	5.70	1.43	0.03	8.68	0.99	25.68	2.18

TABLE 32-continued

Expression of GATA4, CDH1 (E-cadherin), p63, and SOX2 relative to untreated HFF control 4 days after transfecting HFF cells with various gene combinations with potential to reprogram cells into mesoendoderm-like cells.

	GATA4		CDH1 (E-cadherin)		p63		SOX2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
Day 4 Oct4/Sox17/MBD2	23.50	5.39	4.65	4.43	95.23	13.86	18.77	6.94
Day 4 FoxD3/T/MBD2	121.36	11.68	26.85	0.02	2.22	0.04	16.99	4.74
Day 4 FoxD3/Mixl1/MBD2	130.21	21.04	69.19	22.84	4.05	3.56	1.52	0.01
Day 4 FoxD3/Sox17/MBD2	99.49	30.30	6.89	3.69	1.78	0.01	15.19	9.08
Day 4 T/Mixl1/MBD2	110.30	3.55	1.36	0.00	1.36	0.00	6.64	2.25
Day 4 T/Sox17/MBD2	53.19	4.02	2.69	1.86	18.01	0.54	14.21	5.21
Day 4 Mixl1/Sox17/MBD2	16.53	16.50	2.91	2.13	13.44	6.68	10.55	3.27
Day 4 Oct4/FoxD3	66.45	26.34	47.31	47.30	13.87	0.16	23.87	14.31
Day 4 FoxD3/T	68.25	68.00	39.08	29.27	19.45	1.51	19.45	1.51
Day 4 FoxD3/Mixl1	78.18	78.00	21.20	2.31	21.20	2.31	25.10	3.20
Day 4 FoxD3/Sox17	176.45	93.54	15.64	0.60	15.64	0.60	26.78	16.35
Day 4 Oct4/FoxD3/T	12.27	1.26	12.27	1.26	12.27	1.26	12.27	1.26
Day 4 Mixl1/Sox17/FoxA2	85.89	64.52	20.06	20.00	3.67	0.13	13.66	0.66
Day 4 Oct4/FoxD3/T	89.05	50.00	10.40	8.14	1.52	0.02	1.52	0.02
Day 4 Mixl1/Sox17/FoxA2	6.16	6.10	1.23	0.04	1.23	0.04	1.23	0.04

Identification of gene combinations that may induce the formation of Mesendoderm-like cells was investigated by transfection with combinations of Oct4, Sox17, FoxD3, T, Mixl1, FoxA2, and MBD2. As shown in Table 25 and 26, the Relative Expression of CXCR4 and GATA4, both Mesendoderm/Endoderm/Mesoderm markers, appear to be up-regulated in various combinations, most noticeably in FoxD3/Mixl1/MBD2 and FoxD3/Sox17/MBD2. Similarly, FOXA2, a marker for Endoderm and Mesoderm, was up-regulated FoxD3/Sox17-transfected sample, although the expression is still very low. Four days following transfection, SOX17 is still highly expressed in the SOX17-transfected samples (50,000 to 400,000-fold as compared to the untreated HFF sample). The SOX17 gene expression represents leftover plasmid DNA (exogenous SOX17) that still remains 4 days post-transfection, and any endogenous SOX17 expression that may have been induced. Ectoderm markers CDH1, p63 and Sox2 were also up-regulated in some samples (e.g. Oct4/FoxD3/MBD2, Oct4/Sox17/MBD2).

Reprogramming HFFs into Pancreatic Progenitor-Like Cells:

HFF cells were cultured as described in Example I, and transfected using the Nucleofector™® 96-well Shuttle® Device (Lonza) following the procedure described in Example IV. The cells were transfected with various combinations of cDNA clones as described in Table 27. After transfection, the cells were plated on Fibronectin-coated collagen gels and incubated at 37° C., 5% CO_2, 5% O_2. Fibronectin-coated Collagen gel plates were prepared prior to transfection. Rat Tail Collagen I (Gibco) was diluted to 1.125 mg/ml using 10×PBS and distilled water, where 125 µl was added to each well of a 24-well plate and incubated in 37° C. for 40 minutes. After rinsing with 1×PBS, Fibronectin (BD Biosciences) was added on top of the gel at a concentration of 1.9 ug/well. Media was changed every other day according to Table 33. Cells were analyzed at Day 7 by Quantitative Real-time PCR.

TABLE 33

Plasmids and media composition from Day 0 to Day 14

	Media Composition[2]		
Plasmids transfected at Day 0[1]	Day 0	Day 1 to Day 3	Day 4 to Day 14
1 FoxD3, Sox17, Pdx1, MBD2	CDM II + Activin A + HSA	DMEM/F12 + NEAA + ITS + HSA + B27 + EGF + bFGF + Activin A + CHIR99021 + Na Butyrate	DMEM/F12 + NEAA + ITS + HSA + B27 + EGF + bFGF + Retinoic Acid + FGF10 + Cyclopamine + Noggin
2 FoxD3, Sox17, Ngn3, MBD2			
3 FoxD3, Mixl1, Pdx1, MBD2			
4 FoxD3, Mixl1, Ngn3, MBD2			
5 Sox17, Mixl1, Pdx1, MBD2			
6 Sox17, Mixl1, Ngn3, MBD2			
7 FoxD3, Sox17, Mixl1, Pdx1		DMEM/F12 + NEAA + ITS + HSA + B27 + EGF + bFGF + Activin A + CHIR99021 + Na Butyrate + VPA + 5-Aza	
8 FoxD3, Sox17, Mixl1, Ngn3			
9 FoxD3, Sox17, Pdx1, Ngn3			
10 FoxD3, Mixl1, Pdx1, Ngn3			
11 Sox17, Mixl1, Pdx1, Ngn3			

[1]where FoxD3 = pCMV6-XL5-FoxD3, Sox17 = pCMV6-XL4-SOX17, Mixl1 = pCMV6-XL5-MIXL1, Pdx1 = pCMV6-XL5-Pdx1, and Ngn3 = pCMV6-XL5-Ngn3. All clones were purchased from Origene and prepared using the EndoFree Plasmid Maxi Kit (Qiagen).
[2]Supplements added to media at the following concentrations: Activin A (Peprotech, 30 ng/ml), HSA (Baxter, 0.5%), NEAA (Gibco, 1X), ITS (Gibco, 1X), B27 (Gibco, 1%), EGF (Peprotech, 5 ng/ml), bFGF (Peprotech, 10 ng/ml), CHIR99021 (Stemgent, 2 uM), Na Butyrate (Stemgent, 1 mM), VPA (Stemgent, 1 mM), 5-Aza (Sigma, 0.5 uM), Retinoic Acid (Sigma, 2 uM), FGF10 (Peprotech, 50 ng/ml), Cyclopamine (Stemgent, 2.5 uM), Noggin (Peprotech, 50 ng/ml)

Cells were collected on Day 7 and RNA isolation and quantification was performed as previously described in Example I. cDNA was prepared and quantitative real-time PCR was performed as previously described in Example II, except the following Taqman™® Gene Expression Assays (Applied Biosystems) were used:

Gene	Taqman ™ ® Assay ID
GAPDH (housekeeper)	Hs99999905_m1
PPIA (housekeeper)	Hs99999904_m1
FOXA2	Hs00232764_m1
SOX17	Hs00751752_s1
GATA4	Hs00171403_m1
Endo PDX1	PDX1_1201
SOX9	Hs00165814_m1
Ngn3	Hs01875204_s1
NKX2-2	Hs00159616_m1
PAX4	Hs00173014_m1
INS	Hs02741908_m1
CXCR4	Hs00607978_s1

Identification of gene combinations that may induce the formation of Pancreatic Progenitor-like cells was investigated by transfection with combinations of FoxD3, Sox17, Pdx1, Ngn3, Mixl1, and MBD2. FoxA2, a marker for Endoderm and Mesoderm, was slightly up-regulated for the FoxD3/Sox17/Ngn3/MBD2-transfected sample as compared to the GFP mock-transfected control sample. Similarly, CXCR4, also a marker for both endoderm and mesoderm, was slightly up-regulated (3-fold compared to GFP-ctrl) for the FoxD3/Mixl1/Ngn3/MBD2-transfected sample. 7 days following transfection, SOX17 can still be detected for the samples transfected with SOX17 at varying levels (4 to 570-fold up-regulation as compared to the GFP-ctrl). The highest SOX17 expression up-regulation is detected for the sample transfected with Sox17/Mixl1/Pdx1/Ngn3 (570-fold as compared to GFP-ctrl), which may suggest that this gene combination may increase the amount of SOX17 RNA in cells.

Example XIX

Reprogramming Human Adipocytes Derived Stem Cells (ADSC) to Pluripotent-Like Stem Cells (PLSC):

ADSCs (Invitrogen Corporation) were cultured in cell culture flasks with complete StemPro™-43 medium (Invitrogen) at 37° C., 5% CO_2 and the medium was changed 3 times per week. After 3 days in culture cells (passage 5) were trypsinized and counted to be transfected. Cells were transiently transfected with one plasmid: pCMV6-Oct4-2A-Klf4-2A-Nanog, pCMV-Sal14-2A-Oct4-2A-Klf4-2A-Nanog, pCMV-Dax1-2A-Oct4-2A-klf4, pCMV-FoxD3-2A-Oct4-2A-klf4, pCMV-Oct4-2A-Klf4-2A-Sal14, pCMV-MBD2-2A-Oct4-2A-Klf4-2A, pCMV-AGR2-2A-Oct4-2A-Klf4-2A, or Rex1-EF-Oct4-2A-Klf4 (2 µg); or by two plasmids: pEF-Oct4nuc-IRES2-MBD2 with pCMV-Sox2nuc-IREC-Lin28 or pCMV-Klf4nuc-IRES2-Tpt1nuc or pEF-Stella-IRES2-NPM2, using Nucleofector™ as described in Example II. Following the transfection cells were cultured in 6-well plates in suspension with 50:50 ratio of adipocytes complete medium (StemPro™-43) and embryonic stem cells medium (mTeSR1). After two days in culture, cells were re-transfected with the same plasmids listed above and cells were plated in 96 well-plates coated with Matrigel™ (BD Biosciences) in the presence of mTesR complete medium supplemented with thiazovivin (0.5 µM), an ALK-5 inhibitor (SB 341542, Stemgent, 2 µM), and inhibitor of MEK (PD0325901, Stemgent, 0.5 µM). Medium was changed every day and cells were cultured for 22 days at 37° C., 5% CO_2, 5% O_2. Alkaline Phosphatase Detection Kit (AP, Millipore) and immunohistochemistry were performed to analyse the expression of pluripotency markers. ALP staining was performed using AP detection kit (Millipore) according to manufacturer's instructions.

Visual observation of reprogrammed cells was performed by Cellomics™ using a live staining for SSEA-4_{647} (BD Biosciences) and TRA-1-81_{555} (BD Biosciences) starting on Day 6 after transfection and every 5 days thereafter. Reprogrammed colonies of PLSCs, positively stained with SSEA-4 and TRA1-81, was observed only with Plasmid pCMV-Sal14-2A-Oct4-2A-Klf4-2A-Nanog, pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP, pEF-Oct4nuc-IRES1-MBD2 with pCMV-Sox2nuc-IRES1-Lin28, and pEF-Oct4nuc-IRES1-MBD2 with pCMV-Klf4nuc-IRES2-Tpt1nuc. These colonies emerged around Day 6 and maintained in culture up to the end of the study period (Day 22) with a stable morphology. Among the plasmids cited above, pCMV-Sal14-2A-Oct4-2A-Klf4-Nanog and pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP gave the highest number of colonies. Live staining showed that these colonies express typical pluripotency markers, including SSEA-4 and TRA1-81, and further analysis of these colonies showed that the colonies also expressed other ESC markers such as alkaline phosphatase and Oct4. When the cultures were treated with PD0325901 and SB431542 for up to 22 days, a 4-fold improvement in efficiency over the conventional method was obtained following the transfection of ADSCs with pCMV-Sal14-2A-Oct4-2A-Klf4-Nanog and pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP.

Based on the previous study, the highest reprogramming efficiency was observed using pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP and pCMV-Sal14-2A-Oct4-2A-Klf4-2A-Nanog. Another study was designed to ascertain the effect of pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP on the reprogramming efficiency and to investigate the effect of individual pluripotent genes Rex1, Oct4, and Klf4 in different combinations. ADSCs were transfected as above with pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP, pCMV6-XL5-Rex1, pCMV6-XL4-Oct4/pCMV6-XL5-Klf4, pCMV6-XL5-Rex1/pCMV6-XL4-Oct4, or pCMV6-XL5-Rex1/pCMV6-XL5-Klf4. After the second transfection, ADSC were cultured in 96-well plates coated with Matrigel™ for 24 days in the presence of mTeSR1 medium supplemented with SB341542 and PD 0.325901 at 37° C., 5% CO_2, 5% O2. In order to characterize subpopulations of cells after transfection, live staining, immunohistochemistry, and AP staining was used to follow the change in pluripotent markers. 1-5% of total cells transfected with Rex1/Oct4 or Rex1/Klf4 showed a SSEA4$^+$ and TRA-1-81$^+$ phenotype, and this pattern was stable until the end of the study period (Day 22). The observation over time showed that the phenotype of these colonies moved from an early SSEA-4$^+$ phenotype to a late Oct4$^+$/Sox2/Nanog$^+$ phenotype by Day 22, which is closer to the final reprogrammed state of a pluripotent-like stem cell.

Various genes were tested for their effect on reprogramming efficiency towards pluripotent-like cells. ADSC cells were cultured as described in Example IX with 2 days VPA and 5-AZA pre-treatment (1 mM and 0.5 μM respectively) in StemPro™ MSC SFM medium. Cells were transfected using the Nucleofector™® 96-well Shuttle® Device (Lonza) following procedure described in Example IV and using the transfection program EW-104 with the DNA mixes described in Table 34. Following transfection the cells were plated in StemPro™ MSC SFM medium described in example A on Matrigel™ (BD Biosciences) coated 24 well plates and incubated at 37° C., 5% CO_2, 5% O_2. On Day 1, media was changed to a mix of 75% StemPro™ MSC and 25% hES cell medium; the percentage of StemPro™ MSC was decreased every day over four days to have 100% hES cell medium by Day 4. From then onwards the medium was changed every two days. The hES cell medium consisted in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 20% Knockout™ Serum Replacement (KSR, Invitrogen), 1 mM GlutaMAX™, 100 μM Non-essential Amino acids, 100 μM 3-mercaptoethanol and 10 ng/ml Fgf-2. Different inhibitors and growth factors were added through the course of the experiment; these are listed in Table 34. Cells were analysed at Day 7 and Day 14 by immunohistochemistry analysis and at Day 7 by RT-PCR.

TABLE 34

Plasmids and media composition from Day 1 to Day 14.

	From day −2 to day 0	Plasmids transfected at day 0	From day 1 to day 3	From day 3 to day 7	From day 7 to day 14
1	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL5-Sox2 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
2	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL5-FoxD3 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
3	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL5-UTF1 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
4	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL4-DPPA4 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
5	VPA + 5-Aza pre-treated	pCMV6-XL5-Sox2 + pCMV6-XL5-FoxD3 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
6	VPA + 5-Aza pre-treated	pCMV6-XL5-Sox2 + pCMV6-XL5-UTF1 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
7	VPA + 5-Aza pre-treated	pCMV6-XL5-Sox2 + pCMV6-XL4-DPPA4 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium

TABLE 34-continued

Plasmids and media composition from Day 1 to Day 14.					
	From day −2 to day 0	Plasmids transfected at day 0	From day 1 to day 3	From day 3 to day 7	From day 7 to day 14
8	VPA + 5-Aza pre-treated	pCMV6-XL5-FoxD3 + pCMV6-XL5-UTF1 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
9	VPA + 5-Aza pre-treated	pCMV6-XL5-FoxD3 + pCMV6-XL4-DPPA4 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
10	VPA + 5-Aza pre-treated	pCMV6-XL5-UTF1 + pCMV6-XL4-DPPA4 + pCMV6-XL5-MBD2	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
11	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL5-Sox2 + pCMV6-XL5-FoxD3	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
12	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL5-Sox2 + pCMV6-XL5-UTF1	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
13	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL5-Sox2 + pCMV6-XL4-DPPA4	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
14	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL5-FoxD3 + pCMV6-XL5-UTF1	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
15	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL5-FoxD3 + pCMV6-XL4-DPPA4	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
16	VPA + 5-Aza pre-treated	pCMV6-XL4-Oct4 + pCMV6-XL5-UTF1 + pCMV6-XL4-DPPA4	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
17	VPA + 5-Aza pre-treated	pCMV6-XL5-Sox2 + pCMV6-XL5-FoxD3 + pCMV6-XL5-UTF1	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
18	VPA + 5-Aza pre-treated	pCMV6-XL5-Sox2 + pCMV6-XL5-FoxD3 + pCMV6-XL4-DPPA4	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
19	VPA + 5-Aza pre-treated	pCMV6-XL5-Sox2 + pCMV6-XL5-UTF1 + pCMV6-XL4-DPPA4	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
20	VPA + 5-Aza pre-treated	pCMV6-XL5-FoxD3 + pCMV6-XL5-UTF1 + pCMV6-XL4-DPPA4	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 M) + VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium
21	VPA + 5-Aza pre-treated	GFP	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM) +/or − VPA + 5-Aza	StemPro ™/hES medium + ActivinA (30 ng/ml) + CHIR99021 (3 μM)	hES medium

In order to characterize subpopulations of cells after transfection, live staining, immunohistochemistry, and AP staining was performed to follow the change in pluripotent markers. Cells transfected with either Oct4/UTF1/MBD2, Oct4/Dppa4/MBD2, FoxD3/Dppa4/MBD2, Oct4/FoxD3/Dppa4, or Sox2/FoxD3/UTF1 showed positive colonies for TRA1-60, TRA1-81, and SSEA4. This observation indicated that MBD2 generally had no effect by itself on reprogramming towards pluripotent-like cells, except in the case of Oct4/FoxD3/MBD2 transfection. Colonies started to form on Day 7 and continued to form until Day 14 (the end of the study period). These colonies were positive for AP as well.

These results were confirmed by RT-PCR analysis showing up-regulation of Oct4 expression as shown in Table 35. Relative expression for SOX2 was also slightly up-regulation in Day 7 after transfecting cells with Oct4/Foxd3/MBD2. There is also a trend of Sox2 up-regulation following transfection with Oct4/Sox2/Foxd3 and Oct4/Foxd3/Utf1.

TABLE 35

Relative expression of Pluripotent genes after transfecting ADSCs with various combinations of vectors as described in Table 34.

	OCT4		Endogenous SOX2	
	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
#1 Day 7, Oct4/Sox2/MBD2	25.20	1.89	3.89	2.06
#2 Day 7, Oct4/Foxd3/MBD2	11.28	0.13	18.79	0.03
#3 Day 7, Oct4/Utf1/MBD2	2.01	0.20	2.93	1.73
#4 Day 7, Oct4/Dppa4/MBD2	9.68	1.36	1.18	0.15
#5 Day 7, Sox2/Foxd3/MBD2	1.06	0.55	2.68	2.90
#6 Day 7, Sox2/Utf1/MBD2	0.66	0.10	3.36	0.68
#7 Day 7, Sox2/Dppa4/MBD2	0.74	0.00	5.03	4.73
#8 Day 7, Foxd3/Utf1/MBD2	1.31	0.61	4.15	2.92
#9 Day 7, Foxd3/Dppa4/MBD2	0.63	0.02	3.90	2.17
#10 Day 7, Utf1/Dppa4/MBD2	0.96	0.04	4.97	1.92
#11 Day 7, Oct4/Sox2/Foxd3	48.17	1.89	7.68	1.79
#12 Day 7, Oct4/Sox2/Utf1	48.97	6.93	3.71	0.39
#13 Day 7, Oct4/Sox2/Dppa4	32.40	2.74	4.61	2.37
#14 Day 7, Oct4/Foxd3/Utf1	4.30	0.91	9.83	3.03
#15 Day 7, Oct4/Foxd3/Dppa4	4.21	0.11	4.57	0.85
#16 Day 7, Oct4/Utf1/Dppa4	10.29	3.70	3.53	1.63
#17 Day 7, Sox2/Foxd3/Utf1	1.42	0.83	3.32	2.12
#18 Day 7, Sox2/Foxd3/Dppa4	1.19	0.14	3.37	1.23
#19 Day 7, Sox2/Utf1/Dppa4	1.34	0.09	2.33	2.91
#20 Day 7, Foxd3/Utf1/Dppa4	0.72	0.07	2.45	0.27
#21 Day 7, GFP (−VPA/−5aza)	1.02	0.29	1.01	0.17
#22 Day 7, Untransf. ADSC (−VPA/−5aza)	1.25	N/A	0.30	N/A
#23 Day 7, GFP (+VPA/+5aza)	1.01	0.20	1.87	2.23
#24 Day 7, Untransf. ADSC (+VPA/+5aza)	1.45	N/A	0.27	N/A

Reprogramming Efficiency of Defined Pluripotency Factors on HFF after Triple Transfection (One Transfection Every 3 Days)

HFF cells were cultured as described in Example I with the exception of the concentrations of VPA and 5-AZA that were respectively 2 mM and 2.5 μM. Cells were transfected using the Nucleofector™ II Device (Lonza) following procedure described in Example II with the exception of the DNA quantity: 1 μg of each of the 3 plasmids DNA was used. The cells that had been pre-treated with VPA and 5-Aza and the untreated cells were both transfected with a mix of pCMV-Oct4nuc-IRES2-Sox2nuc, pCMV-Klf4nuc-IRES2-Cmycnuc or pCMV-Nanognuc-IRES2-Lin28. Following transfection the cells were plated in the fibroblasts medium described in Example I, supplemented with or without VPA and 5-AZA on Matrigel™ (BDBiosciences) coated on 6-well plates and incubated at 37° C., 5% CO_2. On Day 1 and 2, media was changed to 100% mTeSR1 medium (StemCell Technologies) supplemented with or without VPA and 5-AZA. On Day 3 and Day 6, cells from each condition were detached by incubation in TrypLE™ for 5 min, counted and centrifuged. Cells were retransfected as above and plated on Matrigel™ coated plates in mTeSR1 medium supplemented with or without VPA and 5-AZA. Media was changed daily as described for day 1 and 2. Medium was supplemented in Y27632 (Stemgent, 10 μM) from day 7 to day 14 to promote viability and clonal expansion of potential reprogrammed cells. Cells were analysed at Day 20 using the Alkaline Phosphatase Detection Kit (Millipore) and by immunohistochemistry analysis.

This analysis revealed that after three transfections, three clones were found to be positive for alkaline phosphatase activity and showed a rounded cell/colony morphology. Staining with antibodies against the embryonic stem (ES) cell markers SSEA-4 and TRA-1-81 confirmed that these clones were pluripotent-like. Surrounding HFF cells were negative for these markers. These clones were obtained only in the condition that did not contain inhibitors (i.e.: VPA and 5-AZA). Unexpectedly, no clones were observed for the condition treated with these inhibitors.

Reprogramming of NSLCs into Pluripotency

NSLC and neuronal stem cells derived from BG-01, a human ES cell line that expresses markers that are characteristic of ES cells including SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and OCT-3/4, were reprogrammed into pluripotency. BG-01 cells had previously been cultured in conditions to induce the differentiation towards neural stem cells as described by Chambers S M et al., 2009. NSLCs and BG-01-NSC were cultured in proliferation medium supplemented with FGF (20 ng/ml) and EGF (20 ng/ml). NSLCs and BG-01-NSCs were transfected as previously described in Example II by two episomal vectors, pEF-Oct4nuc-IRES2-MBD2 (NC1) or pCMV-FoxD3-2A-Oct4-2A-Klf4 (F72). Following transfection cells were collected and plated onto uncoated petri-dishes in the presence of Proliferation medium and mTeSR1 medium (50:50) in proliferation conditions at 37° C., 5% CO2. After 48 hours, cells were re-transfected by the same plasmid and plated in 96-well plates coated with Matrigel™ and cultured in the presence of mTeSR1 medium supplemented by the small molecules BIX01294 (Stemgent, 2 μM) and BayK8644 (Stemgent, 2 μM) at 37° C., 5% O_2 for 22 days. Live staining and immunohistochemistry were performed to characterize subpopulations of cells for pluripotency markers.

NSLCs and BG-01-NSCs were positively stained with SSEA-4 starting on Day 7 and maintained until 22 days in culture (the end of the study). Within ten days, cells that were morphologically similar to ESCs were observed and they expressed a wide panel of pluripotency markers, including SSEA-4, TRA1-81, Nanog and Oct4. This study identified another way to get pluripotent-like cells from somatic cells via Neural Stem-Like Cells (NSLCs). The utility of NSLCs could offer multiple advantages for reprogramming towards pluripotent-like cells. For example, obviating the requirement for tumorigenic genes like c-Myc reduces the risk of inducing cancerous cells. For neuroregenerative and neurodegenerative applications these cells could represent an invaluable source of cells to investigate furthermore human pluripotent cell induction, and also represent a potential source of cells for deriving patient-specific multipotent and pluripotent stem cells for modeling human disease.

Example XX

Teratoma Formation Assay in SCID Mice

Transplantation of human pluripotent stem cells (SC) into "severely compromised immuno-deficient" (SCID) mice leads to the formation of differentiated tumors comprising all three germ layers for pluripotent stem cells, resembling spontaneous human teratomas, and specialized tissue for multipotent stem cells. These assays are considered the standards in the literature for demonstrating differentiation potential of pluripotent stem cells and hold promise as a standard for assessing safety among SC-derived cell populations intended for therapeutic applications

After all appropriate animal approvals for the experiment has been obtained, 24 mice were purchased from Charles Rivers, and housed at MISPRO animal facility for one week without any experimentation for adaption to the new environment. One million human NSLCs, normal human neuroprogenitor cells (hNPCs), or human embryonic stem (ES) cells in 100 μl Phosphate buffered saline, calcium- and magnesium-free (CMF-PBS) were injected with a 21-G needle intramuscularly into the right hind limb of the 4-week-old male SCID-beige mice under anesthesia with Ketamine/xylazine (8 mice per group). Following injection, the syringe was aspirated up and down a couple of times in a culture dish containing medium to verify that the cells were injected and not stuck inside the syringe.

The mice were maintained for 12 weeks and monitored for clinical signs and any tumor growth regularly. Any specialized tissue or teratoma growth was monitored by external examination and an increase in the size of the muscle relative to the same muscle on the left hind limb. When a specialized tissue or teratoma was identified, the location and size of the growth was measured (using measuring calipers) and recorded. The specialized tissue or teratoma is usually first identified as a small growth of the muscle size compared to the left control muscle. Animals were monitored weekly until onset of any tumor growth, and daily after tumors appeared. After 12 weeks, the mice were sacrificed by CO_2 euthanasia. Each entire animal was observed for any tumor growth anywhere on the animal, and the injected muscle and the comparable left muscle control were measured (with measuring calipers)(see results table below) and then removed and stored in 4% paraformaldehyde solution for histological analysis. The sizes of the muscles were as follows:

	Left leg (control)		Right leg (treated)	
Treatment	Dorso-ventral width	Lateral width	Dorso-ventral width	Lateral width
Human Embryonic Stem Cells	6.44 ± 0.11	5.03 ± 0.17	6.91 ± 0.15	5.3 ± 0.14
Human Neuroprogenitor Cells	6.60 ± 0.17	5.43 ± 0.15	7.01 ± 0.23	5.58 ± 0.13
Human NSLC	6.85 ± 0.2	5.32 ± 0.14	6.86 ± 0.21	5.33 ± 0.11

Values represent the Average of 8 mice ± the standard error

Measurement of the size of the muscles revealed that all the human embryonic stem cell injected muscles were bigger than the comparable left muscle controls, indicating teratoma growth in the ES cell injected muscles. About half of all the human neuroprogenitor cell injected muscles were bigger than the comparable left muscle controls, while the mice injected with NSLC did not show any difference between the muscles (treated with the cells or not). The mice injected with NSLC did not show any evidence of tumor or teratoma growth.

Example XXI

Transfection of ADSCs by Various Combinations of Genes and Treatment with Small Molecules for Reprogramming to Mesendoderm-Like Cells

Human Adipose-Derived Stem Cells (ADSCs) were purchased from Invitrogen and expanded in complete StemPro™ MSC serum-free medium (Invitrogen on CellStart™ coated flasks (diluted 1:100 in PBS containing Ca^{2+}/Mg^{2+}) at a cell density of 1×10^4 cells/cm^2. ADSCs were cultured as described in complete StemPro medium (Invitrogen) and were pretreated for 2 days prior to transfection with VPA (1 mM) and 5-Aza (0.5 μM). Cells were transfected using the Nucleofector® 96-well Shuttle Device (Lonza) following the procedure described previously. The cells were transfected with various combinations of cDNA clones summarized in Table 36. After transfection, the cells were plated on 0.1% Gelatin-coated plates and incubated at 37° C., 5% CO_2, 5% O_2. Medium was changed every other day according to Table 37. Cells were analyzed at Day 10 by Quantitative Real-time PCR.

TABLE 36

Various combinations of plasmids with potential to transfect the cells towards mesendoderm lineage.

	Day −2 to Day 0	Plasmids transfected at Day 0[1]
1	Untreated	FoxD3, Ngn3, Sox17, MBD2
2		FoxD3, Eomes, Gata6, Mixl1, MBD2
3	Pre-treated	Eomes, FoxD3, Gata6, Mixl1
4	with	Eomes, FoxD3, Gata6, Sox17
5	VPA & 5-Aza	Eomes, Gata6, Mixl1, T
6		FoxD3, Gata4, Mixl1, Sox17
7		Gata4, Gata 6, Mixl1, Oct4
8		Gata4, Mixl1, Sox17, T
9		Eomes, Mixl1, Sox17
10		Eomes, Gata6, Sox17
11		Gata4, Mixl1, Sox17
12		Gata6, Mixl1, Sox17
13		GFP

[1]where FoxD3 = pCMV6-XL5-FoxD3, MBD2 = pCMV6-AC-MBD2, T = pCMV6-XL5-T, Mixl1 = pCMV6-XL5-Mixl1, Sox17 = pCMV6-XL4-SOX17, Gata4 = pCMV6-XL5-Gata4, Eomes = pCMV6-XL4-Eomes, and Gata6 = pCMV6-XL6-Gata6. All clones were purchased from Grigene and prepared using the EndoFree Plasmid Maxi Kit (Qiagen).

TABLE 37

Medium composition from Day −2 to Day 20					
Day −2 to 0	Day 1	Day 2 to day 3	Day 4 to day 7	Day 8 to day 10	Day 11 to day 20
StemPro medium + VPA + 5Aza	CDM II (50%) + IMDM/F12 (50%) + NEAA + ITS + HSA + bFGF + EGF + VPA + Activin A + 5-Aza	IMDM/F12 + NEAA + ITS + HSA + bFGF + EGF + VPA + Activin A + 5-Aza + BMP4	IMDM/F12 + NEAA + ITS + HSA + bFGF + EGF + Activin A + 5-Aza + BMP4	IMDM/F12 + NEAA + ITS + HAS + bFGF + EGF + BMP4	IMDM/F12 + NEAA + ITS + HSA + Exendin-4, nicotinamide

[2]Supplements added to media at the following concentrations: Activin A (Peprotech, 30 ng/ml), HSA (Baxter, 0.5%), NEAA (Gibco, 1X), ITS (Gibco, 1X), EGF (Peprotech, 5 ng/ml), bFGF (Peprotech, 10 ng/ml), VPA (Stemgent, 1 mM), 5-Aza (Sigma, 0.5 uM), BMP4 (Peprotech, 10 ng/ml), Exendin-4 (50 ng/ml, Cat Nb: 24463, Anaspec), and Nicotinamide (10 mM final, Cat Nb: N5535, Sigma).

Cells were collected on Day 10 by detaching with TrypLE, followed by centrifugation at 80×g for 5 minutes. Supernatant was aspirated and the cell pellet was frozen at −86° C. until ready for RNA Isolation. RNA isolation and quantification was performed as previously described: RNA isolation and cDNA was prepared using the High Capacity cDNA RT kit (Applied Biosystems) as per the manufacturer's instructions with a final cDNA concentration of 2 ng/μl. Real-time PCR was then performed for each gene of interest using the FAST PCR master mix (Applied Biosystems) and the Taqman® Gene Expression Assays (Applied Biosystems) listed below:

Gene	Taqman ® Assay ID	Forward Primer Sequence	Reverse Primer Sequence	Probe Sequence
GAPDH (housekeeper)	Hs99999905_m1			
PPIA (housekeeper)	Hs99999904_m1			
FOXA2	Hs00232764_m1			
SOX17	Hs00751752_s1			
Endogenous T	Hs00610073_g1			
GSC	Hs00418279_m1			
CXCR4	Hs00607978_s1			
GATA4	Hs00171403_m1			
CER1	Hs00193796_m1			
CDH1 (E-cadherin)	Hs01023894_m1			
p63	Hs00978340_m1			
SOX2	SOX2_1078-ANY	CCACCTACA GCATGTCCT ACTC	GACCACC GAACCCA TGGA	CTGGCAT GGCTCTTG

-

Ten days following the transfection with various combinations of Oct4, Sox17, FoxD3, T, Mixl1, FoxA2, and MBD2 cDNA, analysis by RT-PCR to investigate the expression of Mesoendoderm and mesendoderm differentiation genes was performed. As summarized in Table 38, the Relative Expression of Sox17 and FoxA2, the markers for Endoderm and Mesodeitu, was up-regulated noticeably in the FoxD3/Mixl1/Ngn3/MBD2-transfected sample, while the expression of mesendoderm differentiation (ex. pancreatic lineage) genes are low indicating an early mesendoderm phenotype. Ten days following transfection, SOX17 is still highly expressed in the SOX17-transfected samples as compared to the untreated ADCs sample that represents leftover plasmid DNA (exogenous SOX17) that still remains 10 days post-transfection, and any endogenous SOX17 expression that may have been induced.

TABLE 38

Relative Expression of the mesendoderm genes FoxA2 and Sox17 after transfecting ADSCs once with various gene combinations with potential to reprogram cells into mesoendoderm-like cells.

	FoxA2		Sox17		Gata4		EndoPdX1		Sox9		Ngn3		Nkx2.2		Insulin	
Day 10	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
Foxd3/Sox17/Ngn3/MBD2	1.13	0.06	44.41	0.51	0.16	0.11	1.13	0.06	0.98	0.05	1.13	0.06	1.13	0.06	1.13	0.06
Foxd3/Mixl1/Ngn3/MBD2	48.03	2.28	11.17	0.58	0.08	0.00	1.71	0.79	0.97	0.08	1.13	0.04	1.51	0.51	1.13	0.04

TABLE 38-continued

Relative Expression of the mesendoderm genes FoxA2 and Sox17 after transfecting ADSCs once with various gene combinations with potential to reprogram cells into mesoendoderm-like cells.

Day 10	FoxA2 Rel. Exp.	FoxA2 Std. Dev.	Sox17 Rel. Exp.	Sox17 Std. Dev.	Gata4 Rel. Exp.	Gata4 Std. Dev.	EndoPdX1 Rel. Exp.	EndoPdX1 Std. Dev.	Sox9 Rel. Exp.	Sox9 Std. Dev.	Ngn3 Rel. Exp.	Ngn3 Std. Dev.	Nkx2.2 Rel. Exp.	Nkx2.2 Std. Dev.	Insulin Rel. Exp.	Insulin Std. Dev.
Foxd3/Mixl1/Pdx1/MBD2	2.36	1.68	0.33	0.07	0.08	0.00	1.19	0.03	1.24	0.08	1.19	0.03	1.19	0.03	1.66	0.63
Eomes/FoxD3/Gata6/Mixl1	3.12	0.06	0.05	0.00	0.22	0.00	3.12	0.06	1.87	0.02	3.12	0.06	3.12	0.06	3.12	0.06
Eomes/FoxD3/Gata6/Sox17	2.64	0.01	137.24	12.01	0.71	0.70	2.64	0.01	1.73	0.02	2.64	0.01	2.64	0.01	2.64	0.01
Eomes/Gata6/Mixl1/T	1.51	0.02	1.37	0.05	0.11	0.00	1.51	0.02	1.46	0.03	1.51	0.02	1.51	0.02	1.51	0.02
FoxD3/Gata4/Mixl1/Sox17	0.78	0.00	84.72	3.22	0.21	0.00	0.78	0.00	1.19	0.12	0.55	0.00	0.78	0.00	0.26	0.00
Gata4/Gata6/Mixl1/oct4	1.35	0.04	3.20	1.20	0.36	0.01	1.35	0.04	1.33	0.10	0.95	0.03	1.35	0.04	0.45	0.01
Gata/Mixl1/Sox17/T	1.20	0.21	228.37	5.78	1.48	0.93	0.41	0.00	0.62	0.02	0.29	0.00	0.41	0.00	0.14	0.00
Eomes/Mixl1/Sox17	1.72	1.41	139.00	7.36	0.20	0.01	0.76	0.05	1.21	0.03	0.54	0.04	0.76	0.05	0.44	0.28
Eomes/Gata6/Sox17	0.57	0.03	573.14	65.66	0.15	0.01	0.93	0.49	0.97	0.05	0.40	0.02	1.55	1.38	0.19	0.01
Gata4/Mixl1/Sox17	1.23	0.02	253.21	45.51	123.32	62.32	1.52	0.21	2.32	0.04	0.58	0.01	2.25	0.65	0.98	0.01
Gata6/Mixl1/Sox17	1.20	0.32	354.21	7.65	0.32	0.01	1.52	0.52	1.23	0.05	0.98	0.12	2.32	0.54	0.78	0.08
ADCs untransf.	0.49	0.02	0.93	0.19	0.49	0.48	0.49	0.02	1.16	0.08	0.34	0.01	0.73	0.33	0.16	0.01

Example XXII

Transfection of ADSCs by Various Combinations of Genes and Treatment with Small Molecules for Reprogramming to Pancreatic Progenitor-Like Cells

Reprogramming ADSCs into Pancreatic Progenitor-Like Cells: Human ADSCs were purchased from Invitrogen and expanded in complete StemPro™ MSC serum-free medium (Invitrogen on CellStart™ coated flasks (diluted 1:100 in PBS containing Ca^{2+}/Mg^{2+}) at a cell density of 1×10^4 cells/cm^2. Cells were transfected using the Nucleofector® 96-well Shuttle® Device (Lonza) following the procedure described previously. The cells were transfected with various combinations of cDNA clones as described in Table 4.

After transfection, the cells were plated on Fibronectin-coated collagen gels and incubated at 37° C., 5% CO_2, 5% O_2. Plates were coated with fibronectin (BD Biosciences) at a concentration of 1.9 μg/well. Media was changed every other day according to Table 39. Following transfection cells will be cultured using high concentration of activin A (50 ng/ml) and BMP (30 ng/ml) to push the reprogramming towards the mesendoderm. To initiate the transition of definitive endoderm to Primitive Gut tube, the medium was supplemented by FGF 10 (Peprotech) and cyclopamine (Stemgent). Thereafter, the gut tube was exposed to Retinoic acid, cyclopamine, and FGF10. In order to stimulate the expression of NGN3 and NKx2.2, the medium was supplemented by exendin 4 and hepatocyte growth factor.

TABLE 39

Plasmids and media composition from Day 0 to Day 20

Media Composition[1]

Plasmids transfected at Day 0	Day −2 to Day 0	Day 1 to 3	Day 3 to 6	Day 7 to 9	Day 10 to 14	Day 15 and over
1- MBD2/Oct4/Sox17 2- MBD2/Oct4/Pdx1 3- Sox17/PDX1/Ngn3 4- MBD2/Ngn3/Sox17 5- MBD2/FoxA2/NGN3 6- MBD2/PDX1/Ngn3 7- Oct4/Sox17/Pdx1/Ngn3 8- Oct4/Pdx1/Ngn3 9- FoxA2/Pdx1/Sox17 10- GFP	StemPro medium	StemPro/RPMI with 17.5 mM glucose, ITS, 1 mM glutamine, 1% HSA + Activin A (50 ng/ml) + BMP4 (30 ng/ml)	RPMI, B27 1%. With 1 mM glutamine, 1% HSA + FGF 10 (10 ng/ml)	DMEM free of glucose/F12 (1:1) + B27 1%, 1% HSA, Retinoic acid (2 nM) Cyclopamine (10 mM), FGF10 (10 ng/ml)	DMEM free of glucose/F12 (1:1) + B27 1%, 1% HSA, Exendin4 (10 nM) & Cyclopamine (10 mM),	DMEM/F12 nicotinamide (10 nM), + Hepatocyte growth factor (20 ng/ml) & exendin4 (10 nM)

[1]Supplements added to media at the following concentrations: Activin A (Peprotech, 30 ng/ml), HSA (Baxter, 0.5%), NEAA (Gibco, 1X), ITS (Gibco, 1X), B27 (Gibco, 1%), bFGF (Peprotech, 10 ng/ml) , , , VPA (Stemgent, 1 mM), 5-Aza (Sigma, 0.5 uM), Retinoic Acid (Sigma, 2 uM), FGF10 (Peprotech, 50 ng/ml), Cyclopamine (Stemgent, 2.5 uM), Exendin-4 (50 ng/ml, Cat Nb: 24463, Anaspec).

Cells were collected on Day 20 to be analyzed by Quantitative Real-time PCR and RNA isolation and quantification was performed as described previously. cDNA was prepared and quantitative real-time PCR was performed with the following Taqman® Gene Expression Assays (Applied Biosystems) used:

Gene	Taqman ® Assay ID	Forward Primer Sequence	Reverse Primer Sequence	Probe Sequence
GAPDH (housekeeper)	Hs99999905_m1			
PPIA (housekeeper)	Hs99999904_m1			
FOXA2	Hs00232764_m1			
SOX17	Hs00751752_s1			
GATA4	Hs00171403_m1			
Endogenous PDX1	PDX1_1201	GGCCCTCTTTT AGTGATACTG GATT	GTAGGAGGGC AGGGATGTG	ACAGCCA CAAACAA CG
SOX9	Hs00165814_m1			
NGN3	Hs01875204_s1			
NKX2-2	Hs00159616_m1			
PAX4	Hs00173014_m1			
INS	Hs02741908_m1			
CXCR4	Hs00607978_s1			

RT-PCR was used to evaluate the expression of pancreatic-related genes from cells after 20 days post-transfection with different combinations of genes (Table 40). This study revealed that FoxA2 (13.12±0.06), Nkx2.2 (23.12±0.06) and Gata4 (5.21±2.36), markers for Endoderm and Mesoderm, were up-regulated for the Sox17/Ngn3/Pdx1-transfected sample as compared to the GFP mock-transfected control sample. Addition of Oct4 to this gene combination increased Gata4 expression as well as Sox17 and endogenous Pdx1 expression, while both gene combinations expressed insulin indicative of islet B-like cells.

TABLE 40

Relative Expression of mesendoderm, pancreatic progenitor, and islet β-cell genes after transfecting ADSCs once with various gene combinations with potential to reprogram cells into pancreatic progenitor like cells (including mesoendoderm-like cells and islet β-like cells).

	FoxA2		Sox17		Gata4		EndoPdx1		Sox9		Ngn3		Nkx2.2		Insulin	
Day 20	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
MBD2/Oct4/Sox17	1.13	0.06	62.97	27.53	0.16	0.11	1.13	0.06	0.98	0.05	1.13	0.06	1.13	0.06	1.13	0.06
MBD2/Oct4/Pdx1	4.72	3.36	10.59	0.29	0.08	0.00	15.91	0.03	0.97	0.08	1.13	0.04	1.51	0.51	1.13	0.04
MBD2/FoxA2/PDX1	43.16	6.34	0.33	0.07	0.08	0.00	14.69	2.26	1.24	0.08	1.19	0.03	1.19	0.03	1.66	0.63
Sox17/PDX1/Ngn3	13.12	0.06	72.37	27.67	5.21	2.38	32.08	14.55	1.87	0.02	97.04	18.77	23.12	0.06	6.12	0.06
MBD2/Ngn3/Sox17	2.64	0.01	137.24	12.01	0.71	0.07	2.64	0.01	1.73	0.02	12.58	3.08	2.64	0.01	2.64	0.01
MBD2/FoxA2/NGN3	72.59	0.02	10.22	0.14	0.11	0.00	1.51	0.02	1.46	0.03	20.33	5.17	1.51	0.02	1.51	0.02
MBD2/PDX1/Ngn3	0.78	0.00	42.36	1.61	0.21	0.00	20.07	6.32	1.19	0.12	59.24	14.07	0.78	0.00	0.26	0.00
Oct4/Sox17/Pdx1/Ngn3	11.35	0.04	139.00	7.36	34.53	17.18	116.68	41.77	1.33	0.10	121.30	4.76	5.35	0.04	6.07	0.06
Oct4/Pdx1/Ngn3	1.72	1.41	1.33	0.36	9.85	5.24	67.42	34.45	1.21	0.03	34.50	2.38	0.76	0.05	1.44	0.28
FoxA2/Pdx1/Sox17	100.57	0.03	143.29	16.42	0.15	0.01	13.19	4.24	0.97	0.05	0.40	0.02	1.55	1.38	0.19	0.01
ADSC untransf.	0.04	0.01	0.21	0.08	0.08	0.00	1.16	0.01	1.02	0.13	1.16	0.01	1.71	0.77	1.16	0.01

Next immunohistochemical analysis was performed to evaluate protein expression in cells transfecting with the different gene combinations. On day 20, the cells were immunostained and examined using the Cellomics™ ArrayScan VTI. Cells were fixed with a 4% formaldehyde/PBS solution for 10 min at room temperature and subsequently permeabilized for 5 min with 0.1% Triton X-100 in 4% formaldehyde/PBS. After two brief washes with PBS, unspecific antibody binding was blocked by a 30 min incubation with 5% normal goat serum in PBS. Then primary antibodies were added in 5% normal goat serum/PBS as follows: Mouse anti-Gata4 (1:100, BD) and mouse anti-FoxA2 (1:200, BD), a marker for endoderm layer. After a 2 h incubation the cells were washed 4 times for 5 min each with 0.1% Tween/PBS. Appropriate fluorescence-tagged secondary antibody was used for visualization; Goat anti-mouse 546 (1:200, invitrogen) prepared in 5% normal goat serum/PBS was used. After incubation for one hour, cells were washed in 0.1% Tween/PBS three times for 5 min each. The DNA stain Hoechst33342 (invitrogen) was used as a marker of nuclei (dilution 1:5000 in PBS, 10 min incubation). Fluorescence images were taken with the Cellomics™ ArrayScan HCS Reader microscopy system. To determine an estimate of the percentage of cells adopting endodermal phenotypes, random fields were selected and for each field the total number of cells (as determined by counting Hoechst stained nuclei) and the total number of cells positive were determined. The cells in the Sox17/Pdx1/Ngn3 and Oct4/Sox17/Pdx1/Ngn3 transfected groups significantly expressed both Gata4 and FoxA2: the number of positive cells was enhanced in the presence of Oct4 as shown in Table 41.

TABLE 41

Percentage of positive cells for Gata4 and FoxA2 at day 20 after transfection of ADSCs with different expression vectors. After transfection cells were cultured in IMDM/F12 supplemented with different type of growth factors and small molecules (as described in Table 4). The percentage of immunopositive cells was determined by the Cellomics ™ ArrayScan HCS Reader.

	Day 20		
	cell count	Gata4%	FoxA2%
GFP	4825	0.21	0.23
untransfected	18646	0.01	0.03
MBD2/Oct4/Sox17	12563	0.17	0.35
MBD2/Oct4/Pdx1	13481	2.61	0.2
MBD2/FoxA2/Pdx1	13418	0.17	0.16
Sox17/Pdx1/Ngn3	15306	11.15	16.9
MBD2/Ngn3/Sox17	19867	1.32	0.31
MBD2/Ngn3/FoxA2	14984	0.33	0.42
MBD2/Ngn3/Pdx1	18613	0.31	10.29
Oct4/Sox17/Pdx1/Ngn3	19515	21.07	30.02
Oct4/Pdx1/Ngn3	6103	0.28	0.49
FoxA2/Pdx1/Sox17	10001	0.65	8.82

Transfection with Pdx1, Ngn3, Sox17 (and Oct4) increased significantly selected pancreas-related genes (Pdx1, Ngn3, NKX2.2, Gata4, FoxA2) indicating a transition from endoderm to a pancreatic fate during this time period. To evaluate pancreatic hormone secretion, pancreatic-like cells were cultured for 50 days, during which time the medium was replaced with fresh medium every two days. We then assayed the supernatant for insulin in the conditioned medium by antigen-capture ELISA kit (Abnova, KA0921) at different time points (day 25 and day 55) and compared to the release of insulin in control cultures. According to the manufacturer's instructions, briefly, 96-well ELISA immunoplates were coated with Anti-insulin (CatNb#) diluted 1/1000 in carbonate buffer (pH 9.7) and incubated at 4° C. overnight. The following day, all wells were washed with TBS-Tween 0.5% before incubation with Block/Sample buffer 1× at room temperature for one hour without shaking. After blocking, standards and samples were added to the plates and incubated and shaken (450±100 rpm) for 2 h at room temperature. Subsequently, after washing with TBS-Tween wash buffer, plates were incubated for 2 h with Anti-Human insulin pAb (1:500 dilution in Block & Sample 1× Buffer) at 4° C. After incubation, plates were washed five times with TBS-Tween 0.5% wash buffer and 100 µl of standard and sample was added in the plates precoated with anti-insulin and incubated for 1 hour at room temperature with shaking (450±100 rpm). Then, plates were washed five times with TBS-Tween 0.5% wash buffer and 100 µl of TMB One Solution was added to each well. Following 10 minutes incubation at room temperature with shaking (450±100 rpm) for the insulin plate, a blue color formed in the wells. After stopping the reaction by adding 100 µl of 1N hydrochloric acid, the absorbance was read at 450 nm on a microplate reader (Synergy 4) within 30 minutes of stopping the reactions. Concentration of released insulin in the supernatants was determined according to the standard curves. ELISA results revealed that insulin was released from cells transfected with Oct4/Sox17/Pdx1/Ngn3, and to a lesser extent in the other groups as summarized in Table 42.

TABLE 42

Quantification of insulin release by pancreatic progenitor/β-like cells that had been cultured for 25 and 55 days after transfecting ADSCs. Insulin release into the medium, at days 25 and 55, was measured by antigen-capture ELISA (Abnova).

Insulin release	Concentration of insulin (µIU/ml) at day 25	Concentration of insulin (µIU/ml) at day 50
MBD2/Oct4/Sox17	0.05	0.07
MBD2/Oct4//Pdx1	1.02	1.87
MBD2/FoxA2/PDX1	1.13	1.11
Sox17/PDX1/Ngn3	2.3	4.32
MBD2/Npt3/Sox17	0.07	1.03
MBD2/FoxA2/NGN3	0.09	1.30
MBD2/PDX1/Ngn3	1.23	1.03
Oct4/Sox17/Pdx1/Ngn3	4.32	7.85
Oct4/Pdx1/Ngn3	2.45	3.21
FoxA2/Pdx1/Sox17	1.02	1.23
ADSC untransfected	0.01	0.03

In addition to increase of gene expression, the transfecting cells were able to release insulin even in low concentrations. Generating reprogrammed **13**-like cell lines that can locally deliver insulin could be used as a method to treat and allow functional recovery from diabetes.

Example XXIII

Transfection of ADSCs by Various Combinations of Genes and Treatment with Different Small Molecules for Reprogramming to Cardiac Progenitor-Like Cells

ADSCs were cultured in StemPro™ MSC serum-free medium (Invitrogen) as previously described, and then transfected with different combinations of cDNA clones as described in Table 43 using the Nucleofector® 96-well Shuttle® Device (Lanza) as described previously. After transfection, the cells were plated on Matrigel-coated plates and incubated at 37° C., 5% CO_2, 5% O_2. Medium was changed every other day according to Table 44. Cells were analyzed at Day 20 by Quantitative Real-time PCR and by Immunohistochemistry.

TABLE 43

Various combinations of plasmids with potential to transfect the cells towards Cardiac Progenitor-like cells lineage.

	Day −3 to Day 0	Plasmids transfected at Day 0[1]
1	Pre-treated	T, FoxD3, Sox17, Mesp1
2	with	Foxd3, Sox17, Mesp1, Nkx2.5
3	VPA & 5-Aza	Foxd3, Tbx5, Baf, Nkx2.5
4		Foxd3, T, Mesp1, Gata6
5		Sox17, Tbx5, Baf, Nkx2.5
6		Foxd3, T, Mesp1, Gata4
7		T, Tbx5, Baf, Nkx 2.5
8		Foxd3, T, Mesp1, Tbx5
9		Mesp1, Tbx5, Baf, Nkx2.5
10		Foxd3, T, Mesp1, Tbx5
11		Foxd3, Sox17, Mesp1, Gata6
12		Foxd3, Sox17, Mesp1, Gata4
13		FoxD3, Sox17, Mesp1, Tbx5
		GFP

TABLE 44

media composition from day −3 to day 20. Media Composition[1]				
Day −3 to 0	Day 1	Day 2 to Day 3	Day 4 to Day 6	Day 7 to Day 20
StemPRo medium + VPA + 5-Aza	IMDM/F12 (50%) + NEAA (1X) + ITS + HAS (5 mg/ml) + VPA + Activin A (30 ng/ml) + CHIR99021 (2 nM)	IMDM/F12 + NEAA + ITS + HSA + FGF8 (10 ng/ml) + 5Aza + VPA + CHIR99021 (2 nM) + Wnt 11 (50 ng/ml) + BMP4 (50 ng/ml)	IMDM/F12 + NEAA + ITS + HSA + FGF8 (10 ng/ml) + FGF (50 ng/ml) + 5Aza + CHIR99021 (2 nM) + Wnt 11 (50 ng/ml) + BMP4 (50 ng/ml)	IMDM/F12 + NEAA + ITS + HSA + FGF (50 ng/ml) + BMP4 (50 ng/ml)

[1]Supplements added to media at the following concentrations: Activin A (Peprotech, 30 ng/ml), HSA (Baxter, 0.5%), NEAA (Gibco, 1X), ITS (Gibco, 1X), FGF8 (Peprotech, 10 ng/ml), bFGF (Peprotech, 50 ng/ml), CHIR99021 (Stemgent, 2 uM), VPA (Stemgent, 1 mM), 5-Aza (Sigma, 0.5 uM), BMP4 (Peprotech, 50 ng/ml)

Cells were collected on Day 20 by detaching with TrypLE, followed by centrifugation at 80×g for 5 minutes. Supernatant was aspirated and the cell pellet was frozen at −86° C. until ready for RNA Isolation. RNA isolation, cDNA preparation and quantitative real-time PCR was performed as previously described.

Gene expression analysis on day 20 following transfection revealed an increased of several Mesoderm markers. Interestingly, three markers for cardiac progenitors cells (Nkx2.5, MyoCD, Tbx5) and three markers of early mesoderm lineage (Gata4, Mesp1, and Meox1) were highly increased after transfecting cells with brachyury (T)/Tbx5/Nkx2.2/Mesp1. Similarly, expression of Mesp1, Tbx5, Nkx2.5, MyoCd, and Meox1 was increased after transfecting the cells with Foxd3/Sox17/Mesp1/Tbx5; while expression of Mesp1, Gata4, Nkx2.5, MyoCd, and Meox1 was increased after transfecting with Foxd3/Sox17/Mesp1/Nkx2.5 and Foxd3/T/Mesp1/Gata4. As shown in Table 45, the Relative Expression of mesoderm and cardiac progenitor cells markers are especially up-regulated in the combination of Mesp1/Tbx5/Nkx2.5/T, Foxd3/Sox17/Mesp1/Nkx2.5 or /Tbx5, and Foxd3/T/Mesp1/Gata4. In general, transfection of ADSCs with a combination Mesp1, FoxD3, Tbx5, Brachyury, Nkx2.5, Sox17 and/or Gata4 increased the expression of mesoderm and cardiac progenitors markers.

In addition, samples were collected at day 20 to evaluate the nature of reprogrammed cells by analyzing the expression of mesoderm markers using Immunohistochemistry analysis for Brachyury and Nkx2.5 according to the methods previously described. Brachyury was significantly expressed in the Mesp1/Tbx5/Nkx2.5/T, Foxd3/Sox17/Mesp1/Nkx2.5 or /Tbx5, and Foxd3/T/Mesp1/Gata4 groups, while all groups expressed Nkx2.5. These cells could represent and invaluable source of cells to investigate furthermore human cardiac cells and also represent a potential source of cells for deriving patient-specific multipotent stem cells for modeling, regenerating, or treating human cardiovascular diseases.

TABLE 45

Relative Expression Gata4, Mesp1, Tbx5, Nkx 2.5, MyoCD, and Meox1, after transfecting ADCs once with various gene combinations with potential to reprogram ADSCs cells into Cardiac Progenitor-like cells.

	Gata4		MESP1		TBX5		NKX2.5		MYOCD		MEOX1	
Day 20	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
GFP-transfected ADCs	1.01	0.16	1.01	0.19	1.00	0.05	1.02	0.27	1.00	0.07	1.02	0.27
Foxd3/Sox17/T/Mesp1	0.02	0.01	6.70	1.18	1.52	0.12	0.94	0.07	1.98	0.13	1.37	0.10
Foxd3/Tbx5/Baf60c/Nkx2.5	0.08	0.03	1.86	0.20	1.59	0.08	24.94	2.97	2.11	0.11	1.17	0.15
Sox17/Tbx5/Baf60c/Nkx2.5	0.62	0.09	3.89	0.10	1.45	0.00	41.72	2.35	0.90	0.02	1.19	0.02
T/Tbx5/Baf60c/Nkx2.5	0.93	0.12	7.59	0.61	1.76	0.12	27.54	2.36	0.94	0.05	1.23	0.19
Mesp1/Tbx5/Baf60c/Nkx2.5	1.12	0.13	31.55	0.91	4.26	0.07	154.76	3.50	1.54	0.06	5.96	1.60
Foxd3/Sox17/Mesp1/Gata6	0.71	0.02	19.59	0.76	1.77	0.15	31.38	1.23	1.44	0.09	3.30	0.68
Foxd3/Sox17/Mesp1/Gata4	0.02	0.00	2.66	0.44	1.45	0.24	0.81	0.24	0.75	0.03	0.65	0.17
Foxd3/Sox17/Mesp1/Tbx5	1.75	3.18	138.28	2.20	15.61	0.87	45.20	14.88	11.06	1.02	4.36	3.45
Foxd3/Sox17/Mesp1/Nkx2.5	17.76	4.34	22.03	0.08	3.03	0.01	95.73	11.68	10.25	2.32	10.54	2.39
Foxd3/T/Mesp1/Gata6	27.10	6.47	3.59	0.42	2.58	0.29	5.11	0.85	21.03	1.02	12.99	1.31
Foxd3/T/Mesp1/Gata4	15.87	6.13	50.20	6.41	2.37	0.18	55.43	6.95	19.86	2.10	4.32	0.15
Foxd3/T/Mesp1/Tbx5	0.83	0.02	8.20	0.48	2.40	0.37	29.25	1.28	0.76	0.08	1.40	0.46
Foxd3/T/Mesp1/Nkx2.5	19.79	2.03	111.35	15.32	2.56	0.35	32.52	12.53	0.29	0.03	24.70	3.12
T/Tbx5/Nkx2.5/Mesp1	18.46	1.06	103.09	22.28	102.53	15.02	96.32	16.60	15.32	5.01	15.73	2.83

Example XXIV

Reprogramming Human ADSCs to Pluripotent-Like Stem Cells (PLSC)

Based on previous results in Example XIX, the highest reprogramming efficiency was observed using pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP (NF10) and pCMV-Sal14-2A-Oct4-2A-Klf4-2A-Nanog (S71). ADSCs (Invitrogen Corporation) were cultured in cell culture flasks with complete StemPro-43 medium (Invitrogen) at 37° C., 5% CO_2 and the medium was changed 3 times per week. After 3 days in culture cells (passage 5) were trypsinized and counted to be transfected. Cells were transiently transfected with one plasmid: pCMV-Sal14-2A-Oct4-2A-Klf4-2A-Nanog or Rex1-EF-Oct4-2A-Klf4-2A-RFP (2 μg) using nucleofector as described in Example II. Following the transfection cells were cultured in 6-well plates in suspension with 50:50 ratio of adipocyte complete medium (StemPro-43) and embryonic stem cell medium (mTesR1). After two days in culture, cells were re-transfected with the same plasmids listed above and cells were plated in 96 well-plates coated with Matrigel (BD Biosciences) in the presence of mTesR complete medium supplemented with thiazovivin (0.5 μM), an ALK-5 inhibitor (SB 341542, Stemgent, 2 μM), and inhibitor of MEK (PD0325901, Stemgent, 0.5 μM). Medium was changed every day and cells were cultured for 22 days at 37° C., 5% CO_2, 5% O_2. Alkaline Phosphatase Detection Kit (AP, Millipore) and immunohistochemistry were performed to analyze the expression of pluripotency markers. ALP staining was performed using AP detection kit (Millipore) according to manufacturer's instructions. Colonies emerged around Day 15 and maintained in culture up to the end of the study period (up to 2 months) with a stable morphology. Live staining showed that these colonies express typical pluripotency markers, including SSEA-4 and TRA1-81, TRA1-60, Oct4, Nanog, Sox2, and E-cadherin. Further analysis of these colonies showed that the colonies present as well other ESC markers such as alkaline phosphatase (ALP, not shown). Supplementing the small molecules PD0325901 and SB431542 with the KSOR medium treated cultures, a 6 fold improvement in efficiency over the conventional method was obtained following the transfection of ADSCs with pCMV-pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP. This pattern did not change up to the 2 month culture period and same positive colonies were observed after transfecting ADSCs with Sal14-2A-Oct4-2A-Klf4-Nanog (not shown). The observation over time showed that the phenotype of these colonies moves from an early SSEA+ phenotype to a late Oct4+/Sox2+/Nanog+ phenotype, which is closer to the final reprogrammed state.

Following the reprogramming of ADSCs cells to Pluripotent-like cells using the two vectors pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP or pCMV-Sal14-2A-Oct4-2A-Klf4-2A-Nanog, a study was performed to examine the differentiation capacity of these cells. Beginning on Day 30, reprogrammed cells were cultured in conditions in order to stimulate the differentiation to embryonic bodies (EBs). These differentiation conditions consisted of placing the cells on uncoated petri-dish in EB Differentiation medium consisting of KSOR medium with FBS 5% without FGF for 45 days. To further differentiate the embryonic bodies, Day 30 EBs were cultured in three different media in order to drive cell differentiation to either ectoderm, mesoderm or endoderm lineages in CDM medium consisting of DMEM/F12 supplemented with 2 mM Glutamine, 0.11 mM 2-mercaptoethanol, 1 mM nonessential amino acids, and 0.5 mg/ml HAS (Yao S et al., PNAS 2006). This CDM medium was further supplemented with different concentrations of cytokines to induce the differentiation towards the three lineages. Ectoderm media consisted of N2/B27-CDM in the presence of Noggin (100 ng/ml) and after 10 days NGF (20 ng/ml) was added to the medium and the cells were grown on laminin-coated plates. Endoderm media consisted of N2/B27 CDM with the presence of Activin A (100 ng/ml) and the cells were grown on Gelatin-coated plates. Mesoderm media consisted of N2/B28-CDM in the presence of Activin A (50 ng/ml) and BMP-4 (50 ng/ml) and the cells were grown on Gelatin-coated plates. Cells that had originally been reprogrammed with pCMV-Sal14-2A-Oct4-2A-Klf4-2A-Nanog differentiated better than cells originally reprogrammed with pEF-Rex1-EF-Oct4-2A-Klf4-2A-RFP which detached from the coated plates. Visual observation of the differentiation of the cells into the three lineages was performed by RT-PCR and Cellomics using the Ectoderm markers (Nestin, GFAP, Beta-tubulin, Sox2), Endoderm markers (GATA4, Brachyury) and Mesoderm markers (GATA4, Brachyury, and Nkx2.5). RT-PCR analysis revealed the Identification of endoderm, mesoderm and ectoderm markers for all 3 embryonic germ layers (Table 46).

TABLE 46

RT-PCR analysis of three embryonic germ layers following the differentiation of PLSCs.

A-Ectoderm	CDH1 (E-cadherin) Rel. Exp.	CDH1 (E-cadherin) Std. Dev.	ASCL1 (MASH1) Rel. Exp.	ASCL1 (MASH1) Std. Dev.	TP63 Rel. Exp.	TP63 Std. Dev.	NOTCH1 Rel. Exp.	NOTCH1 Std. Dev.
S71-transf. ADSC	59180.71	2036.56	70.28	12.35	123114.61	17452.81	5.47	0.19
NF10-transf. ADSC	77944.51	1747.33	48.20	3.27	166929.89	6007.52	6.47	0.12
Mel-2 Undifferentiated, P17	84021.24	178.93	9.80	4.30	10.72	2.96	5.90	0.48
ADSC Untreated Ctrl	1.20	0.93	1.00	0.00	1.00	0.00	1.00	0.03

B-Endoderm	SOX17 Rel. Exp.	SOX17 Std. Dev.	CDX2 Rel. Exp.	CDX2 Std. Dev.
S71-transf. ADSC	9.39	2.21	37.77	5.11
NF10-transf. ADSC	2.33	0.94	21.05	18.59
Mel-2 Undifferentiated, P17	0.77	0.00	4.34	5.05
ADSC Untreated Ctrl	1.00	0.00	1.00	0.00

TABLE 46-continued

RT-PCR analysis of three embryonic germ layers following the differentiation of PLSCs.

	FOXA2		GSC		CXCR4	
C-Mesoderm	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.	Rel. Exp.	Std. Dev.
S71-transf. ADSC	1193.70	51.50	0.85	0.11	512.33	45.62
NF10-transf. ADSC	1662.76	160.28	0.57	0.01	352.65	39.26
Mel-2 Undifferentiated, P17	191.53	18.61	0.01	0.01	105.05	10.62
ADSC Untreated Ctrl	1.00	0.00	1.00	0.06	1.19	0.91

The Expression of TP63 (Ectoderm/Surface Ectoderm) was up-regulated in both the Day 71 S71-transfected sample (123,000-fold) and the Day 75 NF10-transfected sample (167,000-fold) as compared to the ADSC untreated control. The expression of NOTCH1, a marker for the neuroectoderm, was slightly up-regulated in the Day 71 S71-transfected sample (5-fold) and the Day 75 NF10-transfected sample (6-fold) as compared to the ADSC untreated control. Expression of CDH1/E-cadherin, surface ectoderm marker, was up-regulated in both the Day 71 S71-transfected sample (59,000-fold) and the Day 75 NF10-transfected sample (78,000-fold) as compared to the ADSC untreated control. Furthermore, the expression of ASCL1/MASH1, a neural crest marker, was up-regulated in both the Day 71 S71-transfected sample (70-fold) and the Day 75 NF10-transfected sample (48-fold) as compared to the ADSC untreated control.

The expression of definitive endoderm markers were analyzed, such as SOX17, CDX2, and NKX2.1. The expression of SOX17 was up-regulated in the Day 71 S71-transfected sample (9-fold) as compared to the ADSC untreated control. CDX2 gene expression is up-regulated in both the Day 71 S71-transfected sample (38-fold) and the Day 75 NF10-transfected sample (27-fold) as compared to the ADSC untreated control. However, NKX2-1 (Endoderm-Primitive Gut Derivative) was not expressed in any of the 4 test samples at the time points tested.

Markers of Mesoderm were analyzed as well: the expression of T (Mesoderm) was up-regulated in the Day 71 S71-transfected sample (16-fold) as compared to the ADSC untreated control. However the expression of MEOX1, OSR1 (intermediate mesoderm), and FOXF1 (lateral mesoderm) was decreased in both the S71-transfected and the NF10-transfected samples as compared to the ADSC Untreated Ctrl. The expression of GATA4 is up-regulated in both the Day 71 S71-transfected sample (150-fold) and the Day 75 NF10-transfected sample (110-fold) as compared to the ADSC untreated control. The expression of FOXA2 (Mesendoderm, Mesoderm, Endoderm) was up-regulated in both the Day 71 S71-transfected sample (1,100-fold) and the Day 75 NF10-transfected sample (1,600-fold) as compared to the ADSC untreated control. No significant change in GSC (mesoendoderm, Endoderm) expression for both the S71-transfected and the NF10-transfected samples as compared to the ADSC Untreated Ctrl. The expression of CXCR4, a marker for Mesoderm/Endoderm, was up-regulated in both the Day 71 S71-transfected sample (500-fold) and the Day 75 NF10-transfected sample (350-fold) as compared to the ADSC untreated control.

Using immunohistochemistry, the potential of PLSCs to express and differentiate into the three embryonic layers markers was analyzed. Consistent with their hES-like morphology, PLSCs were able to differentiation towards the three lineages: Ectodermal differentiation: Nestin is strongly and widely expressed throughout the experiment. The neural markers GFAP and βIII-tubulin are also expressed, while Sox2 as a neural precursor marker was not detectable at the time points tested, indicating that cells had already differentiated into the neural pathway. At day 12 and 18, many cells co-expressed GFAP and βIII-tubulin. At day 28, however, GFAP expressing cells have almost completely disappeared, while βIII-tubulin positive cells with well formed neurites become detectable. The medium supported neural differentiation, but less astrocyte differentiation, since, especially at day 12 and 18, there are high amounts of GFAP-positive collapsed cells detectable, indicating massive cell death of this type of cells. The omnipresence of the neural markers suggest that differentiation occurs along the neural pathway in this media; although different differentiation lineages could be achieved with different media compositions and growth substrates/growth conditions known in the art. By Day 12 of differentiation, the expression of the stem cell markers Oct4 and Nanog are completely suppressed.

Endodermal differentiation: Large fields of cells that are positive for FoxA2 are detectable indicating endodermal differentiation.

Mesodermal differentiation: Cells treated for mesodermal differentiation cease to express Oct4 and nanog (not shown), indicating that they indeed started to differentiate. The strong and ubiquitous expression of GATA4 and the later appearance of nuclear Nkx2.5 stain indicate that many cells might develop a cardiac profile in the conditions tested. There are some cell clusters that express brachyury staining together with GATA4. As for the Ectoderm and Endoderm differentiation, different media compositions and growth substrates/growth conditions known in the art would promote the acquisition of other differentiation lineages.

Example XXV

Cytotoxic Effects of Human PLSCs

Human Hepatocellular Carcinoma cell line (HepG2, CRL-10741, ATCC) were seeded in a 24 well plate (1×10^4 cells/cm^2) in DMEM with 10% Fetal bovine serum (FBS). PLSCs were harvested using dispase and seeded (1×10^4 cells/cm^2) in transwells (Corning, 0.4 μm) above the HepG2 containing wells. The HepG2 cells were analyzed for cytotoxicity after 24 hours by high-content analysis staining by Propodium Iodide, Yopro1, and Ethidium Heterodimer (cell-impermeable DNA stains that stain only cells with a compromised plasma membrane as occurs in unhealthy cells) along with Calcein (a live stain, marking enzymatic activity in the cytoplasm), Mitotracker Red (marks active, healthy mitochondria) and Cleaved Caspase 3 (a marker for apoptotic or stressed cells). HepG2 cells in the presence of PLSCs had significantly less Propodium Iodide, Yopro1 and Cleaved Caspase 3 staining indicating that the PLSCs had a protective and/or therapeutic/regenerative effect on these cells (Table 47).

TABLE 47

The percentage of Yopro1, Propidium, ethidium Heterodimer, Calcein AM, Mitotracker Red, and Cleaved Caspase-3 positive cells for untreated and PLSCs treated hepatocytes.

		% positive cells					Average intensity
	Number of cells	Yopro1	Propidium Iodide	Ethidium Heterodimer	CalceinAM	Cleaved Caspase3	Mitotracker Red
HepG2 untreated	14539 ± 1359	5.15 ± 0.47	4.57 ± 0.21	5.66 ± 0.02	69.41 ± 1.44	0.95 ± 0.05	26.88 ± 3.52
HepG2, PSLC treated	12498 ± 1454	3.41 ± 0.37	3.85 ± 0.36	5.81 ± 0.33	71.67 ± 1.55	0.63 ± 0.03	27.99 ± 3.74

Example XXVI

Teratoma Formation Assay in Immunodeficient NOD-SCID Mice

Fifty million human NSLCs derived from fibroblast cells (HFF) and fifty millions NSLCs derived from blood cells (CD34+) (according to Examples IV and IX), as well as twenty-five million Human embryonic stem cells (Mel2) were prepared as units of five million cells each embedded with 30% Matrigel (Invitrogen) in 200 μl phosphate buffered saline which were then injected subcutaneously into the back of individual NOD/SCID mice using a 21-G needle within 20 minutes of preparation. Each cell line had been in culture for approximately 1 month prior to the study. Thus 10 mice received an injection of 5 million NSLCs derived from HFF, 10 mice received an injection of 5 million NSLCs derived from CD34+ cells, and 5 mice received an injection of 5 million Mel2 cells. All standard and appropriate animal approval and ethical committee approvals were obtained prior to the commencement of the study.

Although the study was for 3 months, within 1 month of injection one of the mice injected with the Mel2 cells had to be sacrificed due to the size of the teratoma that had formed according to the animal approval protocol. Less than 3 weeks later, the rest of the Mel2 injected mice had to also be sacrificed due to the size of the teratoma that had formed. None of the 10 mice injected with NSLCs derived from fibroblast cells or the 10 mice injected with NSLCs derived from CD34+ blood cells formed any tumors or teratomas, indicating that these NSLCs are safe multipotent stem cells.

Example XXVII

Differentiation of NSLC Derived from HFF, CD34+Cells, and Keratinocytes to Different Neuronal Lineages

To investigate the differentiation potential of NSLCs to different types of neurons, NSLC Neurospheres were dissociated and plated onto laminin/poly-D-Lysine (10 μg/ml; Sigma) coated plates in NeuroCult differentiation medium (NeuroCult Differentiation basal, StemCell Technologies) supplemented with NeuroCult® SM1 Neuronal Supplement and BDNF (20 ng/ml, Peprotech), bFGF (40 ng/ml, Peprotech), FGF 8 (20 ng/ml, Peprotech) and SHH (20 ng/ml, Peprotech) for 30 days. NeuroCult® SM1 (StemCell Technologies) is a standardized serum-free supplement containing antioxidants and retinoic acid; its formulation was developed based on the published supplement formulation identified as B27 but has been optimized to give reproducibly high numbers of functional neurons with minimal glial cell contamination (<1% GFAP+). After one month in culture, the cells were stained with the neuronal marker tyrosine hydroxylase, acetycholine, GABA, and Dopamine. All cell lines stained positive for each of these markers. Immunohistochemistry analysis showed that differentiation medium supplemented with Neurocult SM1 and neurotrophic factors promoted the differentiation of NSLCs derived from HFF, CD34+ cells, and Keratinocytes to dopaminergic, adrenergic, and Gabanergic neurons.

This study showed that NSLCs derived from fibroblast, keratinocyte, or blood cells are capable to differentiate towards different types of neurons in the appropriate differentiation conditions. The specific neurons could be used to treat diseases where such neurons are affected or have been lost, for example, dopaminergic neruons in Parkinson's disease. Other types of multipotent stem cells and progenitor cells prepared according to the methods in the previous examples would be expected to reprogram or differentiate more along certain pathways with the appropriate media and supplements, growth substrate and growth conditions known in the art.

Example XXVIII

Comparison of Expression of Specific Genes in Human HFFs, NSLCs Created from the HFFs, and Human Primary Neuroprogenitor Cells

The expression of selected genes and proteins in NSLCs created from HFFs according to Example IV were determined. Total RNA was extracted from cells, using Trizol following manufacturer's recommendation. Briefly, cells were examined for the expression of different genes associated with pluripotency: SOX2 (460 bp), OCT4a (172 bp), OCT4b (169 bp) and NANOG (276 bp); early neural markers: SOX2 (460 bp), NES (327 bp), CD133 (200 bp), PAX6 (431 bp) and ASCL (220 bp); Notch signaling; NOTCH1 (126 bp), NOTCH2 (475 bp), HES1 (314 bp) and HESS (265 bp); neurotrophic factors: GDNF (389 bp) and BDNF (743 bp) and astrocyte marker GFAP (650 bp) and markers of neurons: NFL (284 bp), NFM (333 bp), NFH (316 bp), SYN (289 bp), NSE (292 bp) and MAP2 (321 bp). Positive controls used were NT2 cells (human NT2/D1 teratomacarcinoma cell line (ATCC), NT2-derived neurons (NT2-N) and astrocytes (NT2-A) and SH-SY5Y cells were appropriate. No template control was used as negative control for every gene examined.

NSLCs had the same expression profile as human primary neuroprogenitor cells (NP; Lonza), namely in terms of expression of Sox2, Oct4b, Notch1, Notch2, Hes1, Hes5, Nestin, CD133, Pax6, Ascl1 (Mash1), NFL, NFM, NSE and Map2, but additionally the of the neurotrophic factors GDNF and BDNF. The HFF cells that the NSLCs were created from also expressed Oct4b (Oct4b is a spicing variant of Oct4a (a stem cell marker) which expression is not limited to stem cells), Nestin, some of the notch signaling genes and neuronal markers, making it significantly easier to reprogram than adult dermal fibroblasts that do not express this genes (thus, for example, Nestin is important to add to the reprogramming cocktail when reprogramming adult fibroblasts to NSLCs).

Next immunohistochemistry was used to confirm that the encoded protein of some of the above expressed genes was present in the cells. Cells were fixed with 65% Ethanol and 0.15M NaCl for 20 minutes and stained for the following proteins: SOX2, NES, GFAP, MAP2, NCAM and RBPJ (green). Phase and Hoechst (blue) counter stain was also preformed. NT2 and NP cells were used as comparison and positive controls. NSLCs expressed intense staining for Sox2, Nestin, GFAP, Map2, and RBPJ with only a small amount of NCAM detected. NP cells had an identical staining pattern to the NSLCs except slightly less intense staining especially for Sox2 and Nestin. The NT2 cells only expressed Sox2, Nestin and RBPJ at appreciable levels. The HFFs from which the NSLCs were created from only expressed Nestin and RBPJ at appreciable levels.

Example XXIX

Potential of NSLCs for CNS Therapeutic Applications

NSLCs prepared from HFFs according to Example IV were tested for their ability to form functional cell-cell communication through gap junctions, which is an important characteristic for therapeutic potential when implanted into the CNS. Dye coupling experiments showed functional cell-cell communication in NSLC cells through gap junctions. A single NSCL cell was pre-loaded with two dyes [DiI (red) and calcein AM (green)] and plated on an unlabeled layer of NSCL cells. Calcein readily transferred from the donor cells to a large population of receiving cells after 3 hours in vitro, confirming gap junctional intercellular communication among NSCL cells.

Next NSLCs were tested for the expression of synaptotagmin (a synaptic vesicle protein) and MAP2 (a neural differentiation associated marker) since NSLCs have some unique characteristics over native neural stem/progenitor cells including expression of some growth factors as well as a more significant expression of some neural differentiation markers while maintaining the ability to remain in a stem cell like state. NSLCs were found to readily express synaptotagmin and Map2.

Next NSLCs were tested for their tolerance to glutamate and NMDA to determine their potential robustness in areas of significant ischemia, trauma, and neurodegeneration in the CNS. NSLCs maintained a healthy neuronal state in the presence of glutamate (200 mM), a characteristic not observed in the presence of NMDA (25 mM), indicating an ability to form synapses upon neuronal differentiation.

Next the NSLCs were tested for their ability to attach and survive on 3D scaffolds; in this case PGA scaffolds. NSLCs were found to readily attach to the PGA scaffolds designed for implantation into the brain. Carboxyfluourodiacetate (CFDA) confirms optimal cell survival (>93%) 24 hours after cells were plated on the scaffold. NSLCs showed robust attachment and survival to the surface for at least one week in vitro. Furthermore, Neurites grew along the length of each PGA fiber, following the pattern of the scaffold, indicating that NSLCs can be grown on 3D scaffold and structures useful for implantation into humans or animals, or for in vitro modeling.

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Headings are included herein for reference and to aid in locating certain sections These headings are not intended to limit the scope of the concepts described therein under, and these concepts may have applicability in other sections throughout the entire specification Thus, the present invention is not intended to be limited to the embodiments shown herein but is to be accorded the widest scope consistent with the principles and novel features disclosed herein.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the present invention and scope of the appended claims.

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We claim:

1. An in vitro human neural multipotent or unipotent cell possessing all of the following characteristics:

i) has the ability to proliferate for more than 50 population doublings, and has an average population doubling time of less than 3 days;

ii) is not a cancerous cell;

iii) is stable and not artificially maintained by forced gene expression and may be maintained in standard neural stem cell media;

iv) can differentiate to a progenitor, precursor, or somatic cell;

v) has the characteristics of a neural stem cell, neural precursor cell, neural progenitor cell or neuroblast;

vi) does not exhibit uncontrolled growth, teratoma formation, and tumor formation in vivo;

vii) expresses one or more markers of a multipotent, unipotent or somatic cell not characteristic of a neural stem cell, neural precursor cell, neural progenitor cell or neuroblast;

viii) is capable of maintaining telomerase activity through to at least about 30 population doublings;

ix) is derived from the reprogramming of a somatic cell, a progenitor cell or a stem cell that exhibits at least a transient increase in intracellular levels of at least one reprogramming agent; and

x) wherein the cell comprises at least one polypeptide or an expression vector encoding at least one polypeptide selected from the group consisting of:

Musashi1 (Msi1);

Ngn2;

Msi1 and Ngn2;

Msi1 and methyl-CpG binding domain protein 2 (MBD2);

Ngn2 and MBD2;

Msi1, Ngn2 and MBD2;

Achaete-Scute Homolog 1 (Ascl1);

Msi1, Ngn2 and Ascl1;

Msi1, Ngn2, MBD2 and Ascl1;

Sox2;

Msi1, Ngn2 and Sox2; and

Msi1, Ngn2, MBD2 and Sox2;

wherein the expression vector is transiently expressed.

2. An in vitro human neural multipotent or unipotent cell, wherein the cell possesses all of the following characteristics:

i) expression of one or more neural stem cell markers selected from the group consisting of Sox2, Nestin, Glial Fibrillary acidic protein (GFAP), Msi1, and Ngn2;

ii) is capable of being cultured in suspension or as an adherent culture;

iii) is capable of proliferating without the presence of an exogenous reprogramming agent for over 1 month;

iv) expression of one or more markers selected from the group consisting of synaptophysin, synaptogamin1, synaptotagmin, GAP-43, microtubule associated protein 2, neural-specific tubulin, NCAM and markers for neurotransmitters upon neuronal differentiation;

v) expression of one or more markers of a multipotent, unipotent or somatic cell not characteristic of a neural stem cell, neural precursor cell, neural progenitor cell or neuroblast;

vi) capable of maintaining telomerase activity through to at least about 30 population doublings;

vii) has the ability to proliferate for more than 50 population doublings, with an average population doubling time of less than 3 days; and

viii) wherein the cell comprises at least one polypeptide or an expression vector encoding at least one polypeptide selected from the group consisting of:

Musashi1 (Msi1);

Ngn2;

Msi1 and Ngn2;

Msi1 and methyl-CpG binding domain protein 2 (MBD2);

Ngn2 and MBD2;

Msi1, Ngn2 and MBD2;

Achaete-Scute Homolog 1 (Ascl1);

Msi1, Ngn2 and Ascl1;

Msi1, Ngn2, MBD2 and Ascl1;

Sox2;

Msi1, Ngn2 and Sox2; and

Msi1, Ngn2, MBD2 and Sox2;

wherein the expression vector is transiently expressed; and possesses one or more of the following characteristics:

ix) forms neurospheres in the neurosphere-colony formation assay;

x) is capable of dividing every 36 hours at low passage;

xi) is capable of differentiation into one or more of a neuronal cell, an astrocyte, or an oligodendrocyte;

xii) has decreased expression of telomerase and one or more neural stem cell markers upon differentiation;

xiii) has one or more morphological processes characteristic of immature axons and/or dendrites and greater than one cell diameter in length;

xiv) expresses at least one marker for a neurotransmitter selected from the group consisting of dopamine, acetylcholine, and gamma aminobutyric acid (GABA);

xv) is capable of releasing one or more neurotrophic factors;

xvi) is negative in a tumor colony forming assay;

xvii) is negative for tumor growth in Severely Compromised Immuno-Deficient (SCID) mice;

xviii) is negative for teratoma growth in SCID mice;

xix) is capable of significantly improving one or more functional measures after placement of an adequate number of the cells into the void in a brain ablation model;

xx) is capable of significantly improving or maintaining one or more functional measures after injecting an adequate number of the cells into an Experimental Allergic Encephalomyelitis (EAE) mouse model;

xxi) is capable of improving one or more functional measures more significantly than hNPCs in CNS injury or neurodegenerative models; and

xii) is derived from the reprogramming of a somatic cell, a progenitor cell or a stem cell that exhibits at least a transient increase in intracellular levels of at least one reprogramming agent.

3. An in vitro human neural somatic cell that possesses all of the following characteristics:

i) expresses at least one neural-specific antigen selected from the group consisting of neural-specific tubulin, microtubule associated protein 2, NCAM, and a marker for a neurotransmitter;

ii) is capable of releasing one or more neurotrophic factors;

iii) is not a cancerous cell;

iv) is stable and not artificially maintained by forced gene expression and may be maintained in standard neural media;

v) is capable of growing axons and/or dendrites that are more than one cell diameter in length;

vi) has the characteristics of a neural cell;

vii) does not exhibit uncontrolled growth, teratoma formation, and tumor formation in vivo;

viii) expresses one or more markers of a multipotent, unipotent or somatic cell not characteristic of a neural cell;

ix) wherein the cell is derived from the reprogramming of a somatic cell, a progenitor cell or a stem cell that exhibits at least a transient increase in intracellular levels of at least one reprogramming agent; and

x) wherein the cell comprises at least one polypeptide or an expression vector encoding at least one polypeptide selected from the group consisting of:

Musashi1 (Msi1);

Ngn2;

Msi1 and Ngn2;

Msi1 and methyl-CpG binding domain protein 2 (MBD2);

Ngn2 and MBD2;

Msi1, Ngn2 and MBD2;

Achaete-Scute Homolog 1 (Ascl1);

Msi1, Ngn2 and Ascl1;

Msi1, Ngn2, MBD2 and Ascl1;

Sox2;

Msi1, Ngn2 and Sox2; and

Msi1, Ngn2, MBD2 and Sox2;

wherein the expression vector is transiently expressed.

4. A plurality of isolated cells of claim **1**, **2** or **3** wherein the cells are organized within a three dimensional structure.

5. A pharmaceutically-acceptable composition comprising cells according to any one of claims **1**, **2** and **3**, for transplantation into a patient in need thereof.

* * * * *